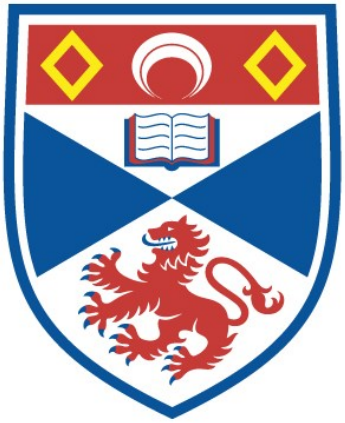
CAROTENOID COLOUR AS A CUE TO HEALTH IN HUMAN SKIN

Audrey Joan Henderson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



2017

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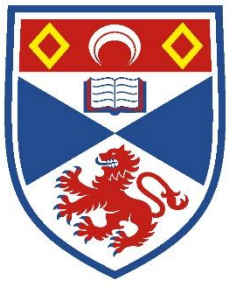

University of
St Andrews

This thesis is submitted in partial fulfilment for the degree of PhD in Psychology

at the

University of St Andrews

August 2016

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I, Audrey Joan Henderson hereby certify that this thesis, which is approximately 33,000 words in length, has been written by me, and that it is the record of work carried out by me, or principally by myself in collaboration with others as acknowledged, and that it has not been submitted in any previous application for a higher degree.

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Collaboration statement

Throughout the experimental chapters in this thesis, I have used the pronoun “we” in addition to “I”. This work is my own under the support of my supervisor in terms of hypotheses; experimental design, analysis and conclusion; however, the Perception Lab is an inherently collaborative environment. The plural pronoun reflects the fact that if/when published, the following experiments would carry multiple authorship and is used in keeping with intellectual honesty.

The work contained in Chapter 5 is partially based on work submitted to a peer-reviewed academic journal. This article, along with the list of contributing authors, is identified at the beginning of Chapter 5. Collaborators and co-authors based in Karolinska Institutet are credited with the design of Study 6, and associated data collection. The study was designed to investigate objective and subjective response to activation of the immune system in healthy volunteers. Data were shared to allow independent analysis of skin colour change. The hypotheses, analysis and interpretation of results are my own, under support of my supervisor.

Acknowledgements

This thesis would not have been possible without the help and support of many people.

First, thank you to Dave Perrett, for supervising this thesis with an unwavering enthusiasm. Thanks also to the Eastbio Doctoral Training Partnership for providing funding; and to Karen Spencer and Simon Powis for their time and effort as second and co-supervisors.

Many thanks are due to friends and fellow PhDers during my time in the Perception Lab: Iris Holzleitner, Carlota Batres, Jing Ma, Sean Talamas, and Martha Lucia Borras; thank you all for lab dinners, science chats, and for keeping me positive. Lesley Ferrier and Dengke Xiao, thank you too for practical and technical support.

Past Perception Lab members have made the lab what it is today and I would like to extend thanks also to Ross Whitehead, for passing the baton (or spectrophotometer); and Fhionna Moore, for introducing me to Evolutionary Psychology, but also providing honest words of encouragement when times were tough.

I would like to express my deepest gratitude to my family for always believing in me, and for providing words of motivation and encouragement on demand. Special thanks are given to my dad, Ellice Walker, who fostered my interest in science, and to my mum, Elizabeth Walker, who taught me the perseverance needed to pursue it this far.

Finally, thank you to my husband, Ewan, who has waited patiently for me to complete this work and to Luna and Morris, my faithful writing companions.

Abstract

Carotenoids are red-yellow plant based pigments. When consumed, they contribute to human skin yellowness which in turn is perceived as healthy and attractive looking. In many non-primate species, carotenoids colour sexually selected ornaments, signalling health. This thesis explores the relationship between skin yellowness and aspects of human health to test the hypothesis that carotenoid colouration of skin acts as a cue to health beyond diet.

Chapter 2 presents a demonstration that a modest change in carotenoid intake can lead to a favourable change in appearance. Chapter 3 investigates the relationship between skin yellowness and a number of health related risk factors. Findings show that that psychological stress varies with skin yellowness (independent of fruit and vegetable intake) both between and within participants. Chapter 4 investigates the relationship between prior symptoms of infectious illness (e.g. cold and flus); finding that symptoms experienced during the prior eight weeks are related to skin yellowness between subjects and also within (marginally). More recent symptoms of illness (i.e. the prior week) were not reflected in skin yellowness. The final empirical chapter explores skin colour and plasma carotenoid changes with experimentally induced sickness. Plasma carotenoids were found to reflect baseline skin yellowness and showed a reduction in response to sickness but this reduction was not reflected in skin yellowness. Skin colour did change in a manner consistent with changes in blood perfusion and oxygenation status. A follow-up perceptual study confirmed that this change can reliably inform judgements of health.

Taken together, findings suggest that skin yellowness is related to aspects of health beyond diet (i.e. psychological stress and prior illness) on a timescale of weeks but not days; likely reflecting the time taken for carotenoids to reach the skin. Theoretically, findings support the hypothesis that carotenoid colouration of human skin is a cue to health.

Contents

Chapter 1: General introduction

1.1. What are carotenoids?

Carotenoids are a group of fat-soluble, red, orange and yellow plant based pigments which help to protect against damage from the sun's UV rays. They give colour to autumn leaves and numerous fruits and vegetables; for example: tomatoes, carrots, squash, peppers, and mangos (Tanumihardjo, 2013). Carotenoids are also highly abundant in dark green leafy vegetables, although their colour is not seen here because they are masked by green chlorophyll. In animals, carotenoids cannot be synthesised de novo, and must be obtained from the diet. Once consumed, carotenoids will colour sexually selected ornaments in a number of bird and fish species; these brightly coloured ornaments (e.g. feathers, fins or beaks) will attract mates by signalling the individual's health status (see section 1.2). In humans, carotenoids contribute towards the visible colourations of skin (see section 1.3) and such colouration is perceived as healthy and attractive looking (see section 1.4). Whilst carotenoids are known to have health promoting properties (see section 1.5); it is not known whether carotenoid colouration of human skin reflects the health status of an individual, as has been shown in other species. This thesis takes an evolutionary psychology approach to understanding why carotenoid colouration of human skin is deemed attractive and if this preference reflects a valid cue to health.

An evolutionary approach to understanding facial preferences assumes that they serve an adaptive function (Little, Jones, & DeBruine, 2011); that they afford some benefit to the individual holding these preferences which allow genes for the behaviour to be passed onto the next generation. Presumably, being able to accurately assess the current or general health of others can afford a number of potential benefits. Direct benefits, for example could be gained in terms of disease avoidance (DeBruine, Jones, Tybur, Lieberman, & Griskevicius, 2010). By choosing not to mate with unhealthy looking individuals, you would avoid catching contagious infections. Selecting a mate who is in good general health also increases the chance that your partner will be around and able to assist with raising offspring; additionally, you may be securing indirect benefits in terms of parasite resistant genes for offspring.

This evolutionary approach has been applied to help understand many universal preferences in facial features. For a full review of facial features that influence judgements of health, and their validity, i.e. the evidence that they reflect actual health, see Appendix 1: Henderson et

al., (2016). This approach prompts us to question whether carotenoid colouration of human skin is a valid reflection of health, and if so, what specific information the cue provides.

The literature review which follows describes first the evidence to date of carotenoid signalling in non-primate species and possible mechanisms. I will then go on to summarise the evidence for two premises that must be true if carotenoid colouration has evolved as a cue to health in humans: firstly, that carotenoids colour human skin, and secondly, that this colour is perceived as healthy and attractive. The final section reviews links between carotenoids and health in humans before setting the scope of empirical work contained within this thesis.

1.2. Carotenoid signalling in other species

Carotenoid colouration of ornaments has been linked to greater mating success in fish (Bourne, Breden, & Allen, 2003), butterflies (Davis, Cope, & Smith, 2007) and birds (Hill, 1990; Zuk, Thornhill, Ligon, & Johnson, 1990). Brighter individuals also have more viable offspring, and higher mate retention, as shown in great tits (Helfenstein, Losdat, Saladin, & Richner, 2008). With regard to sexual signalling, it is often the case that females do the choosing and males display. However, in the case of carotenoid ornamentation, some species show bidirectional selection with both males and females both selecting for brightly coloured partners (Amundsen & Forsgren, 2001; Massaro, Davis, & Darby, 2003). Whilst carotenoid ornaments are accepted as a sexually selected signal amongst many bird and fish species; the biological underpinnings of what the ornaments actually signal is far from agreed (Svensson & Wong, 2011; Vinkler & Albrecht, 2010) but much studied.

Early on, Endler (1980), suggested that carotenoid ornaments demonstrate an individual's foraging ability; stemming from the knowledge that carotenoids are obtained exclusively from the diet. This idea was later shown to not account for all variation in carotenoid colouration of feathers in greenfinches (Karu, Saks, & Hõrak, 2007). The saturation of feather colour increased when the birds were supplemented with carotenoids but amongst both supplemented and placebo birds, the colour of wild grown feathers (produced before experimental intervention) correlated strongly with those grown during the experimental phase. This suggests that feather colour reflected individual differences irrespective of carotenoid availability.

When diet is held constant, carotenoid ornaments have been shown to vary with instances of ill health. For example, when injected with sheep red blood cells (to illicit a non-specific immune response), the beaks of male blackbirds showed a decrease in colour saturation measured three weeks later. Furthermore, birds that showed a stronger immune response to the sheep red blood cells, showed a greater decrease in beak colouration (Faivre, Grégoire, Préault, Cézilly, & Sorci, 2003). Male red jungle fowl infected with intestinal worms during infancy also show duller combs and paler feathers at sexual maturity (Zuk et al., 1990);. whilst reducing nematode worms with treatment has been shown to increase comb redness (Martínez-Padilla, Mougeot, Pérez-Rodríguez, & Bortolotti, 2007). House finches too, when experimentally infected with a natural parasite show a reduction in feather brightness and colour saturation, from three weeks post infection (Brawner, Hill, & Sundermann, 2000; Hill, Farmer, & Beck, 2004). In the wild, intensity of carotenoid ornaments also correlate negatively with parasite load. In House finches, the intensity of avian pox infection has been shown to negatively correlate with plumage redness (Thompson, Hillgarth, Leu, & Mcclure, 1997) and red grouse with brighter combs also have fewer parasites (Martínez-Padilla et al., 2007).

In addition to changes in health status, carotenoid ornaments also appear to provide an indication of condition or resistance to illness; for example, house finches with redder feathers were found to be more likely to survive a natural epidemic (Nolan, Hill, & Stoehr, 1998). Furthermore, brighter Zebra finches (resulting from a high carotenoid diet) were able to mount a stronger innate immune response relative to paler individuals when provoked (Blount, Metcalfe, & Birkhead, 2003), as were male greenfinches with brighter feathers (Aguilera & Amat, 2007) and male mallard ducks with brighter bills (Peters, Denk, Delhey, & Kempenaers, 2004).

To date then, the evidence that carotenoid colouration of ornaments can reflect aspects of health beyond diet is clear, but the physiological mechanisms underpinning these cues are less well understood. The knowledge of the physiological role that carotenoids play led to a number of new theoretical explanations of how carotenoids may be employed to honestly signal health. Carotenoids have the chemical potential to act as antioxidants, donating electrons to unstable molecules (reactive oxygen species or free radicals) that occur as a result of normal metabolism and external stressors (Møller, Wallin, & Knudsen, 1996), when there are insufficient antioxidants to deal with free radicals, this state is known as oxidative stress, and leads to DNA, protein and lipid damage. This understanding led to an “antioxidant

role" hypothesis, positing a trade-off, whereby only individuals who do not need carotenoids to deal with reactive oxygen species can use them to colour ornaments (Lozano, 1994).

Whether or not carotenoids do have a biologically significant role as antioxidants *in vivo* has been questioned (Costantini & Møller, 2008; Young & Lowe, 2001) but it has been argued that even if they do not, as long as they become colourless when they lose electrons, they are able to signal an individual's overall antioxidant capacity. Two variations of this idea are (1) the "sparing" hypothesis which suggests that carotenoids spared from their antioxidant role by an effective antioxidant network can instead be used for ornaments (Hartley & Kennedy, 2004) and (2) the "protection" hypothesis which suggests that carotenoids when used as antioxidants become dangerous (in their oxidised form) and exacerbate the state of oxidative stress; but individuals with an efficient endogenous antioxidant network (i.e. antioxidants beyond carotenoids) can afford to carry and display carotenoids in ornaments (Vinkler & Albrecht, 2010).

Carotenoid ornaments have also been proposed to signal immune function although this too can be tied to their function as antioxidants. The immune system is a contributor to oxidative stress as reactive oxygen species are utilised to kill invading pathogens (de la Fuente, 2002). It has therefore been suggested that only individuals with an efficient antioxidant network can mount a strong immune response and not suffer collateral damage as a result (von Schantz, Bensch, Grahn, Hasselquist, & Wittzell, 1999). Finally, carotenoids also have other immunostimulatory effects that are independent of oxidative stress. Carotenoids have been linked to an enhancement of signalling between immune system cells and expression of immune-related genes (Pérez-Rodríguez, 2009). These functions have again led to trade off hypotheses suggesting that healthier individuals would need fewer carotenoids for immunological functions, allocating them instead to ornament expression (Lozano, 1994; von Schantz et al., 1999).

In more recent years, it has been proposed that signal honesty does not require resource trade-offs or fitness costs but that carotenoid colouration of individuals could reflect the overall condition of an organism if tied to the efficiency of basic cellular processes (Hill, 2011; Johnson & Hill, 2013). Overall condition is a term used to describe the ability of an individual to maintain optimal performance and encompasses the somatic state of the organism (e.g. current health, age, energy reserves) as well as genetic and epigenetic variation (Hill, 2011). If each of these factors influence basic cellular function, such as the

efficiency of cellular respiration (the process of extracting energy from nutrients), and this is directly linked to carotenoids ornamentation (as argued by Johnson and Hill, (2013)), then carotenoids colouration would be reflecting overall condition or mate quality of an individual, which would encompass both immune function and antioxidant status. Under this model carotenoid colouration can be expected to reflect environmental factors such as exposure to disease or stress; as well as heritable factors such as resistance to diseases or the effectiveness of endogenous antioxidant defences.

It is important to note that all of these hypotheses permit carotenoids to act as a signal for current or general health and it is not clear whether selection for high carotenoid displays is based on direct (avoidance of disease) or indirect (good genes) benefits (Adamo & Spiteri, 2009; Scott, Pound, Stephen, Clark, & Penton-Voak, 2010). Finally, it is also important to note that the proposed hypotheses may not be mutually exclusive, for example, a good forager may be able to compensate for heritable (or environmentally induced) immune deficiency.

Whilst carotenoid signalling has been extensively studied in birds and fish species there is a distinct lack of research concerning carotenoid signalling in primate species. Almost all primate species are excessively covered in hair or fur (with the exception of humans) which is coloured by melanin rather than carotenoids. Nevertheless, many primates do have exposed areas of skin around the face or rump where colour plays an important role in social and sexual signalling (Bradley & Mundy, 2008). In such species, variation in red colouration is most often studied and usually communicates short-term changes in blood flow effected by stress, sex hormones or social interactions. Primates absorb carotenoids from their diet and likely utilise these pigments for their antioxidant properties (Cutler, 1984), in humans at least, excess carotenoids are also deposited in the skin where they act as a UV protectant (see section 1.3). Given that primates have the necessary trichromatic colour vision to see variation in carotenoid colour (Bradley & Mundy, 2008) and that colour plays an important role in social and sexual signalling for these species, further research investigating the role of carotenoids in colour signalling is warranted.

1.3. Carotenoids in human Skin

In humans, carotenoids, once consumed and absorbed, can reach the skin via one of two mechanisms: the first is via diffusion from the blood (Darvin et al., 2008); the second is

through sebaceous or sweat glands, escaping in sebum and sweat before being partly reabsorbed by the stratum corneum (the outer most layer of skin) (Alaluf, Heinrich, Stahl, Tronnier, & Wiseman, 2002) The most common carotenoids in human skin are lycopene and beta-carotene respectively (Scarmo et al., 2010).

Individuals will vary in their ability to absorb carotenoids (Britton, Liaaen-Jensen, & Pfander, 2009), once in the skin though, carotenoid content can be assessed by one of three methods. For a long time tissue biopsies were the only viable option (Mayne et al., 2010) with high-performance liquid chromatography (HPLC) being used to identify, isolate and quantify carotenoids. More recently, Raman spectroscopy has been developed as a non-invasive alternative to assessing skin carotenoids (Darvin, Gersonde, Meinke, Sterry, & Lademann, 2005; Mayne et al., 2010). This method is based on the understanding that when any molecule is hit by light, a very small amount of that light will interact with the molecule, causing it to vibrate and re-emit light in a different form. The way in which the light is changed is a function of the chemical structure of that particular molecule, for example the number and position of carbon double bonds. By looking for this specific signature of shift in light associated with carotenoids and measuring its intensity we can gain a measure of concentration of carotenoids present (Hata et al., 2000). The final method, light reflectance spectrophotometry, involves measuring light returned at a range of wavelengths when light of a known spectrum is shone on the skin. Values returned refer to the skin as whole. For this reason, it is more difficult to estimate quantities of specific compounds within. At least one lab group however, have applied mathematical models to light reflectance data in order to estimate levels of carotenoids (Stahl et al., 1998).

Studies to date using all of these methods have confirmed two main findings: increasing carotenoid consumption (through fruit and vegetables or supplements) is correlated with increased skin carotenoid levels; and secondly, skin carotenoid levels correlate with plasma levels. Stahl et al. (1998) demonstrated both of these points by providing 12 women with daily beta-carotene supplements for 12 weeks. They found that this led to a significant increase in skin carotenoids, as estimated using light reflectance spectrophotometry and purpose designed software. They also found strong correlations between the estimates of carotenoids in the skin and levels of carotenoids in the plasma. Although the strength of these correlations were location specific, strong associations were found in the palm of the hand and the forehead (*r* values were .94 and .89 respectively). Similarly Stephen, Coetzee and Perrett (2011) found that supplementation of beta-carotene for eight weeks in 10 participants

significantly increased skin yellowness as measured by light reflectance spectrophotometry; and that this yellow change was consistent with the known absorption spectrum of carotenoids (in wavelengths 400-500nm).

Dermal levels of carotenoids also reflect differences in fruit and vegetable consumption between participants; with individuals who eat more fruit and vegetables having more yellow skin, as measured by light reflectance spectrophotometry (Stephen et al., 2011; Whitehead, Re, Xiao, Ozakinci, & Perrett, 2012). Melanin was controlled for in these studies (by statistically controlling for variation in skin lightness) to negate the suggestion that individuals who eat more fruit and vegetable may also spend more time outdoors. Rerksuppaphol and Rerksuppaphol (2006) also found a relationship between fruit and vegetable consumption and skin carotenoids, as measured by Raman Spectroscopy. Participants who consumed low amounts of fruits and vegetables per day (less than two portions per day) had significantly lower skin carotenoid levels relative to those consuming moderate (two to three portions per day) or high (four of more portions per day) amounts. Finally, Mayne et al. (2010) found that across 28 participants, skin carotenoids measured by Raman spectroscopy correlated significantly and highly with skin carotenoids levels assessed from biopsies (r=.66) and with plasma carotenoid levels (r=.62).

So far, the evidence summarised has demonstrated that carotenoids are present in human skin, correlate with fruit and vegetable consumption and reflect plasma levels. However, for skin carotenoids to act as a cue to the bearer's health, differences in skin carotenoid content must also be visible. One way to test this is to measure skin colour in CIE L*a*b* colour space, which is designed to quantify colour in a manner that is consistent with the human perceptual system. In CIE L*a*b* colour space, colour and is specified along three axes: L* represents darkness/lightness on a scale from 0 (black) to 100 (white); a* represents a scale from green (negative values) to red (positive values); and b* represented a scale from blue (negative values) to yellow (positive values) (see Figure 1). Values on the a* and b* scale are unlimited and higher values represent more saturated (i.e. intense) colouration, with values close to 0 appearing dull. In the context of skin colour, only positive values are relevant and so a* and b* will often be referred to simply as "redness" and "yellowness" respectively (Stamatas, Zmudzka, Kollias, & Beer, 2004).

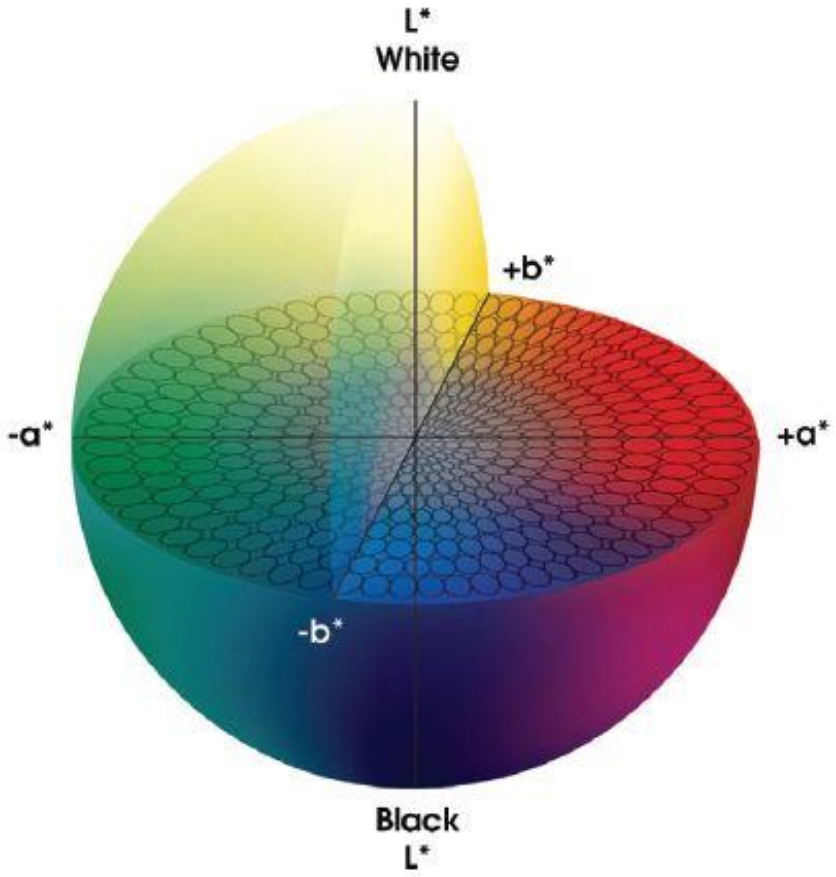

Figure 1: Graphical representation on CIE L* a* b* colour space.

In CIE L*a*b* colour space Alaluf et al (2002) measured skin colour of 22 participants using a tristimulus chromameter (an instrument which measures colour along the 3 axes of L* a* b* colour space) . In addition, they measured skin carotenoid levels using Stahl et al's methods (described above and which were demonstrated to correlate highly with plasma levels). What they found were strong correlations between skin carotenoids and the b* component of skin colour (i.e. skin yellowness) but no relationship between skin carotenoids and L* (lightness) or a* (redness) values. Skin carotenoids were not exclusively responsible for all of the variance in yellowness which was also explained in part by variation in melanin levels; and the strength of correlation between carotenoids and yellowness varied with the site at which they were measured. The strongest relationships between skin colour and carotenoid content were in the palm of the hand, forehead, inner arm and back (correlation coefficients in these regions varied from .75 to .85). These strong correlations allowed Alaluf and colleagues to conclude that carotenoids do contribute significantly to the coloration of human skin; and that skin colour may act as a means of assessing dietary carotenoids status.

Other studies have subsequently replicated the finding that carotenoid supplementation measurably influences the yellowness of skin not only amongst individuals of Caucasian ethnic background (Pezdirc et al., 2015; Stephen et al., 2011; Whitehead, Re, et al., 2012) but also of African and Asian ethnic background (Coetzee & Perrett, 2014; Stephen et al., 2011; Tan, Graf, Mitra, & Stephen, 2015; Whitehead, Re, et al., 2012).

According to colour detection thresholds published elsewhere (Re, Whitehead, Xiao, & Perrett, 2011; Tan & Stephen, 2013), the magnitude of colour change measured in these studies is sufficiently large to influence perception of skin colour (see also Chapter 2 for further discussion and an assessment of health judgments in response to a modest change in carotenoid intake). In the section that follows, perceptual studies investigating judgments of health or attractiveness as a function of skin yellowness and specifically carotenoids colouration are reviewed.

1.4. Yellow is attractive and healthy

Perceptions of health and attractiveness are guided, amongst other features, by skin colouration. When given the opportunity to optimise the apparent health of facial stimuli, individuals will choose to increase the redness, yellowness and lightness of skin (Stephen, Law Smith, Stirrat, & Perrett, 2009). Skin yellowness in particular has also been shown to correlate with judgements of attractiveness in natural, manipulated images (Henderson et al., 2016; Scott et al., 2010; Stephen et al., 2012).

The yellow appearance of human skin is influenced by two main pigments; in addition to carotenoids, melanin also increases skin yellowness. Melanin is produced in response to sunlight and also varies genetically according to ethnic background.

In an attempt to understand which of these pigments drive perceptions of health, Stephen and colleagues conducted a perceptual experiment where they digitally altered the appearance of faces to demonstrate how they would look with an increase in melanin or carotenoids (Stephen et al., 2011). The researchers measured the colour difference between sun exposed and non-sun exposed areas of skin to create a melanin transformation. They also measured skin colour after eight weeks of beta-carotene supplementation to create a carotenoid transformation. These were matched in magnitude of total colour change and applied to a set of 51 Caucasian faces. In 1D and 2D slider trials (whereby participants could move a cursor

horizontally across the computer screen to manipulate skin colour along one or more dimensions) participants increased both carotenoid and melanin colour to make faces look more attractive, however, in both cases carotenoid colour was increased significantly more; suggesting a greater preference for this pigment. The finding that carotenoid colouration of human skin is preferred over melanin, has been replicated in a forced choice design also (Lefevre & Perrett, 2014).

The preference for carotenoid colouration in Caucasian faces has also been demonstrated using a colour transformation derived from natural variation in fruit and vegetable consumption (Whitehead, Re, et al., 2012). This particular study found that a change in as little as two portions per day was detectably different and a difference as small as three portions per day was deemed healthier looking. The preference for skin yellowness, and specifically carotenoid colouration of skin, is not limited to Caucasian stimuli or raters and has also been demonstrated in stimuli of faces from African-American and Asian ethnic backgrounds (Stephen et al., 2011; Whitehead, Coetzee, Ozakinci, & Perrett, 2012).

Finally, the preference for carotenoid colour is thought to be specific to faces when compared with scrambled images (Lefevre, Ewbank, Calder, Hagen, & Perrett, 2013). In a forced choice experiment, displaying identity matched faces which differed only in the degree of carotenoid colouration of skin; participants consistently preferred the face with more carotenoid colour. When these same faces were scrambled to retain all colour information but no longer look like a face, there was no consistent preference. The authors suggest that a face specific preference for carotenoid colour may be because here it holds important biological information. What biological information might carotenoid colouration of human skin hold? Surely, carotenoid colouration of skin will reflect carotenoid intake (as seen in section 1.3) because carotenoids are obtained exclusively from the diet but carotenoids also have physiological functions in the human body as antioxidants and immunostimulants and so colouration could be a function of both carotenoid intake and carotenoid expenditure related to maintaining health (as we saw was true of other species in section 1.2).

The prior two sections have summarised evidence that carotenoids colour skin and that this colouration will affect judgements made by an observer (in terms of health or attractiveness). The present thesis aims to explore whether these judgements are well justified, in other words, if carotenoid colouration of skin is a valid indication of some aspect of health. If so, carotenoid colouration can be considered, in evolutionary terms a cue or a signal. Cues are

any feature that can be used to infer a hidden quality (Maynard Smith and Harper 2003). A cue is a signal only if it evolved specifically for the purpose of communicating information. Signals evolve from cues and unless there is some obvious cost to carrying the observed trait (e.g. increased predation risk) then it is often not clear whether that trait is a cue or signal. This thesis does not test why carotenoid colouration of skin evolved only whether it is a valid reflection of health. Any confirmatory findings would therefore support the notion that carotenoid colouration of skin is a cue (but not necessarily a signal) to health. The next section reviews known links between carotenoid consumption or measured levels of carotenoids and human health.

1.5. Carotenoids and health

Epidemiological studies have shown that higher plasma levels of carotenoids are associated with lower risk of coronary heart disease and a number of cancers (Rao & Rao, 2007). Since plasma carotenoid levels are correlated with fruit and vegetable intake, it is impossible to know whether the protective effects come from the carotenoids themselves or other constituents of the foods containing them. However, to some extent this limitation is irrelevant for the purpose of the present thesis. Whether health benefits are gained directly from carotenoids, or fruits and vegetables does not matter because carotenoids will be consumed, and will therefore colour the skin in both cases. In other words, carotenoid colour of skin could cue benefits from carotenoids, or from other substances ingested along with the carotenoids.

The evidence linking fruits and vegetables to health is indisputable, a number of large scale epidemiological studies have demonstrated an association between high fruit and vegetable intake and lower risk of many diseases, including cardiovascular disease (Boeing et al., 2012; Rao & Rao, 2007; Steffen et al., 2003), various types of cancer (Boeing et al., 2012; Key, 2011) dementia, osteoporosis, macular degeneration, asthma (Boeing et al., 2012), upper respiratory infections (Li & Werler, 2010), and all-cause mortality (Genkinger, Platz, Hoffman, Comstock, & Helzlsouer, 2004; Steffen et al., 2003).

Placebo controlled supplementation studies (whereby participants are given powdered fruit or vegetable extracts in a capsule and compared to a group given placebo capsules) have also had largely positive results. For example in decreasing blood pressure (Engelhard, Gazer, & Paran, 2006), reducing symptoms of the common cold (Roll, Nocon, & Willich, 2011); and

boosting immune function (Nantz, Rowe, Nieves, & Percival, 2006), measured as an increase in circulating T cells and a reduction in DNA damage in lymphocytes.

Carotenoid supplementation studies have been more controversial and led to mixed results. Some have shown benefits, for example improved immune function (Chandra, 1992; Hughes et al., 1997) whilst others have not, including two large scale clinical intervention studies of beta-carotene and alpha-tocopherol (Blumberg & Block, 1994), or beta-carotene and retinol (Omenn et al., 1996) in smokers. Intervention trials have their own limitations, especially around the doses administered which are usually much larger than those naturally obtained from the diet. Carotenoids given in supplementation form will also be more easily and quickly absorbed by the body than carotenoids obtained through food.

The health effects of carotenoids then, are still not completely understood, but there is strong evidence to support the idea that fruit and vegetable consumption will both benefit health and colour skin in a manner that is deemed healthy in appearance.

Raman Spectroscopy (as introduced in section 1.3) has in recent years been used to investigate how skin carotenoids vary in response to illness and stressors. Across 75 participants, skin carotenoids measured by Raman spectroscopy showed no difference by age, weight, alcohol intake, gender, race, education or skin tone but did correlate with total carotenoid intake assessed by food frequency questionnaire (r=.52) and fruit and vegetable intake (r=.39) (Mayne et al., 2010). Consistent with some of these findings another study of 29 participants found higher skin carotenoids (measured by Raman spectroscopy) amongst individuals who reported high consumption of fruits and vegetables relative to those who report moderate or low consumption but no difference in skin carotenoids amongst under and over-weight individuals (Rerksuppaphol & Rerksuppaphol, 2006).

Maeter and colleagues measured skin carotenoids by reflectance spectroscopy (a more recent technique in quantifying skin carotenoids similar conceptually to Raman spectroscopy and validated against it (Darvin et al., 2012)). The authors measured skin carotenoids in a small sample of nurses (n=7) before and after morning or night shifts (Maeter et al., 2013). The authors note that the drop in measured carotenoids (55%) is greater following a night shift than that following a morning shift (8.5%). This study presented a series of observational case studies with descriptive statistics rather than a controlled experiment. A limitation was that not all nurses were recorded in all conditions and there was variation in the time of day

that measures were taken. For example, in one nurse, the before and after night shift measurements were taken 3 days apart. For two other nurses, measures "before night shift" were taken during the morning of a previous day shift and "after night shift" measures were taken during the evening of a second night shift. The study presents important evidence that skin carotenoids can vary within a short period of time (days or hours) but the evidence that working night shifts, relative to morning shifts causes a greater reduction in carotenoid levels is limited. A follow-up study would benefit from controlling the time of day at which measures are taken and the length time between measures, for example immediately before and after a single morning or night shift; ideally both conditions would also follow a day off. Power for the experiment could be increased by employing a strictly within subject design whereby all participants are measured in both conditions. Such an experiment could provide stronger evidence as to whether night shift stress reduces skin carotenoids.

Another study reported that skin carotenoids (both lycopene and beta-carotene) measured by Raman spectroscopy are lower in smokers and higher in vegetarians (Darvin et al., 2005). Carotenoids were measured at the palm, inner forearm, forehead and backs. From a sample of 28 volunteers, 6 vegetarians were compared to 6 heavy smokers and an undisclosed number of non-smokers. Full demographics of this sample were not provided with reference to smoking and vegetarianism, and so it is unknown whether all 28 volunteers are represented in this comparison (with some being represented twice, i.e. non-smoking vegetarians) or whether a subset of volunteers have been selected.

The same research group have also published a year-long investigation of variation in skin carotenoids, measured daily by Raman spectroscopy amongst 10 volunteers. Results of the study show a clear group level increase in skin carotenoids during summer and autumn months when fresh seasonal fruits and vegetables would be more readily available (Darvin et al., 2010). The authors also suggest that stressful life events precede a decrease in skin carotenoids. These findings are largely exploratory and descriptive. For each volunteer, researchers calculated average carotenoid levels during winter-spring and summer-autumn months, any values falling one standard deviation beyond these means were considered outliers and daily questionnaires were consulted to retrospectively explain the "untypically strong increases or decreases". The authors provide examples of decreases in carotenoids following events such as "fatigue", "food poisoning", "illness", "party at night", and "alcohol consumption". This study is pivotal in exploring links between skin carotenoid and health, and provides pointers toward lifestyle factors worthy of further consideration. However,

follow up work employing a more systematic approach to testing the relationship between stressors and skin carotenoid measures is necessary to confirm these links.

An investigation of skin carotenoid levels in response to endurance exercise found that following 30 minutes of running or cycling, skin carotenoids at the palm and forehead showed a significant drop (Vierck et al., 2012). However, the sample size was small (n=6 for each exercise condition), and standard deviations were large. Statistical analysis was performed comparing baseline measures to those immediately after exercise and to individual's maximum decrease (which varied in time point by individuals). Individual's trends over time were not presented; which would have demonstrated whether maximum drops selected for analysis represented one clear drop or otherwise. Interestingly, in the cycling condition, carotenoids fell only after moderate intensity exercise and not high intensity. This result is difficult to understand if carotenoids are thought to be lost with exercise; as we would expect greater intensity of exercise to show a stronger and clearer drop in carotenoids. The effect of exercise on skin carotenoid levels is therefore unclear, though the authors suggest that skin carotenoids fall in response to an exercise session.

In summary, whilst studies of skin carotenoids using Raman spectroscopy have provided some illustrations that skin levels of carotenoids may relate to smoker status and various life stressors (night shift work, physical exercise, alcohol consumption and illness) much of the work is exploratory and in its infancy. Studies tend to report a small number of case studies with only descriptive analysis of selected data. Further work employing a more systematic approach to investigating the effect of lifestyle and health upon skin carotenoids is necessary.

1.6. Summary and scope of the thesis

The evidence presented in this chapter demonstrates sufficient cause to suggest that the carotenoid colouration of human skin could act as a cue to health. In birds and fish, carotenoid based ornaments are known to reflect health or condition of the individual, as well as mating success. In humans, consumption of fruits and vegetables is associated with a host of health benefits and an increase in fruit and vegetable consumption leads to changes in skin colour. Finally, these changes are judged as more attractive and healthy looking. Studies using Raman spectroscopy point towards the possibility that skin carotenoids fluctuate with factors beyond fruit and vegetable consumption but are lacking in rigor and also do not speak to the appearance of human skin. Even if these changes in skin carotenoids with non-dietary

aspects of health are valid, it is not known whether these variations in skin carotenoids would be of great enough magnitude to influence the appearance of human skin. What remains to be tested then is whether carotenoid colouration of human skin is a valid indication of health status beyond fruit and vegetable consumption and this gap forms the overarching theme of the present thesis.

Coloration of human skin is well placed to serve as an indicator of current condition because it may change over a short period of time. In exploring why a preference for carotenoid colouration of skin exists it is important to note that evolutionary consequences could arise if carotenoid colouration were a reliable indication of either current health or general health and both these possibilities will be explored in the current thesis.

The first experimental chapter presents results from a carotenoid supplementation study, testing whether an achievable change in diet can affect skin colour in a sample of individuals who vary in constitutive skin tones; and whether these changes are perceived as healthy. This study aims to demonstrate effects which the current literature has shown are true in theory. Chapter 3 explores the relationship between carotenoid colouration of skin and a range of individual differences which are known to be risk factors for health, these risk factors can also be considered indicators of condition. A cross-sectional observation study is employed to test whether carotenoid colouration of skin reflects smoker status, alcohol consumption, body fat, psychological stress, and exercise habits. These same factors are then explored again within a repeated measures study. Chapter 4 investigates signs and symptoms of ill health and asks whether, as seen in the ecology literature, instances of ill health are reflected in carotenoid colouration. Finally, Chapter 5 explores how plasma carotenoids and skin colouration respond in the short term (over a period of 8 hours) to an activation of the immune system.

In all experimental chapters, skin colour was measured as the dependent variable using a, spectrophotometer (model: Konica Minolta CM-700d, d65 illuminant 8° illumination angle, specular component excluded).

The measurement of skin colour here, as opposed to measurement of skin carotenoids is deliberate because the aim of the thesis is to examine whether varying levels of skin carotenoids provide a cue to the health status of others; and it is colouration of the skin that is directly perceived by observers.

It is noted however that whilst L*, a* and b* values provide an objective measure of the perception of skin colour; they are not well placed as an analytical tool for explaining physiological mechanisms (Stamatas et al., 2004).

In addition to carotenoid content of skin, b* values are also influenced by melanin content. Unlike carotenoids though, the presence of melanin will darken the colour of skin (Alaluf, Atkins, et al., 2002; Stamatas et al., 2004; Stephen et al., 2011). Additionally, changes in the volume of hemoglobin have been shown to influence b* values (Stamatas et al., 2004). Therefore, throughout this thesis, b* (i.e. skin yellowness) is considered the dependent variable, but variation in skin lightness and skin redness will also be considered (either as covariates or in separate analysis) in order to account for melanin or blood as potential mediating chromophores (molecules responsible for colour). Where the term "carotenoid colouration of skin" is used, this will refer to variation in skin yellowness which is independently related to the variable of interest (i.e. health).

Chapter 2: Carotenoid supplementation

2.1. Chapter outline

This first empirical chapter aims to provide a "proof of concept" with regard to the perceptual benefits gained through increased carotenoid consumption. Prior work has demonstrated that carotenoids colour skin, and that in theory a modest change in dietary intake can provide perceivable benefits, but such effect has not yet been demonstrated in a single self-contained study. Here, 10 young adult participants with a range of natural variation in skin tone took part in a carotenoid supplementation study. Volunteers were provided with two additional portions of vegetables (one pepper and 250ml of carrot juice) per day over a six week period. An analysis of skin colour confirmed that during the course of the experiment skin yellowness increased but skin lightness and redness remained unchanged. A median split to compare individuals with the lightest and darkest constitutive skin tones showed no difference in terms of the degree to which skin yellowness increased. Photographs taken before and after the intervention were used to test judgments of health in a forced choice experiment with 136 independent raters. Photographs were displayed either as raw images or with only the colour change applied to a copy of the baseline images to isolate this cue. In both instances, the image after supplementation was selected as healthier on 60% of trials and significantly above chance. This two part study confirms that naturally obtained variation in carotenoid intake can have positive benefits in terms of perceived health.

2.2. Introduction

Dietary carotenoids are known to accumulate in the skin and measurably influence skin yellowness. If carotenoids are to accurately inform judgements of health made by others, then their influence on skin colour must also be both perceivable and preferable. Each of these premises have been tested empirically with respect to lightly pigmented individuals, but gaps remain in understanding whether all premises are true across a range of skin tones. Furthermore, although studies have shown that carotenoids increase skin yellowness, and separately that increasing skin yellowness improves judgements of health; no study has demonstrated the perceptual benefits associated with an achievable increase carotenoid consumption in a single sample of individuals.

Supplementation studies using carotenoid capsules have shown an increase in measured yellowness of human skin in Caucasian (Stephen et al., 2011) and African skin (Coetzee & Perrett, 2014), although, in the latter study, only measures at the palm location remained significant after statistical correction for multiple comparisons. Variation in fruit and vegetable consumption has also been associated with differences in skin yellowness between Caucasian individuals (Pezdirc et al., 2015; Stephen et al., 2011) and an increase in dietary consumption of fruits and vegetables has been associated with a concurrent increase in skin yellowness in Caucasian (Pezdirc et al., 2015; Whitehead, Re, et al., 2012) and Asian skin (Tan et al., 2015).

The preference for skin yellowness has been demonstrated in African skin, both amongst natural, unmanipulated images (Coetzee et al., 2012; Stephen et al., 2012) and when participants have the opportunity to manipulate facial colour to maximise apparent health (Stephen et al., 2011). However, in the case of natural images, where studies find that skin yellowness correlates with judgements of attractiveness in African skin (Coetzee et al., 2012; Stephen et al., 2012), there are high levels of collinearity between skin lightness, redness and yellowness, which all loaded on a single factor for skin colour. Skin colour then, was shown to significantly influence judgements of attractiveness but whether this was driven by variation in carotenoid levels is not obvious. In other perceptual work, when participants are allowed to manipulate colour for maximum apparent health, there is a consistent finding that chosen levels are greater than measured changes from supplement studies. Stephen et al., (2011) allowed African participants to digitally alter the levels of skin yellowness in African faces and found that, similar to results in Caucasian individuals, participant chose to increase

yellowness by 6 CIE units to maximise apparent health; however, there is no evidence to date that a colour change of this magnitude can be achieved through carotenoid consumption. In supplementation studies, the maximum group level change in skin yellowness reported is 1.67 CIE units in African skin (Coetzee & Perrett, 2014), 1.26 CIE units in Caucasian skin (Stephen et al., 2011) and 3.25 CIE units in Asian skin (Tan et al., 2015).

Amongst lightly pigmented individuals, this point is less contentious as Whitehead and colleagues went on to demonstrate that the measured colour change associated with a difference in 3 portions of fruits and vegetables per day over a 6 week period was sufficient to influence judgements of health (Whitehead, Re, et al., 2012). However, this study showed only that in theory, a modest change in diet could favourable influence judgements of health. Judgements of health were made upon facial stimuli which were digitally altered to demonstrate the skin colour change associated with fruit and vegetable consumption. This colour alteration however was derived from differences between 15 high and 15 low carotenoid consumers in a prior cross-sectional study, rather than a change within individuals in response to carotenoid consumption. The colour applied may therefore have been confounded by other between subject differences such as constitutive skin colour or sun exposure.

Whitehead and colleagues went on to demonstrate the perceptual benefits of carotenoid colouration using a colour transformation based on within-subject changes in skin colour which were associated with natural variation in fruit and vegetable consumption over a 6 week period (Whitehead, Ozakinci, & Perrett, 2013, 2012). In these studies, the carotenoid transform was applied to Caucasian faces and raters were able to manipulate colour to maximise health. On average, participant chose to add colour equivalent to 6 (Whitehead et al., 2013) or 7 additional portions of fruit and vegetable per day to maximise apparent health. This magnitude of change in fruit and vegetable consumption is ambitious but attainable and these studies provide further strong evidence that natural variation in skin carotenoid levels could influence judgements of health. Again however, carotenoid colour measures are taken from one group of individuals and applied to another, furthermore colour is applied universally across facial skin and this may not best represent the distribution of colour change with carotenoid consumption. These studies also do not speak to the smallest unit of change in diet needed to influence judgements of health.

Other perceptual studies have sought to determine minimal colour difference thresholds at which a perceivable difference exists or upon which judgements will be made. Tan and Stephen (2013) for example, presented Asian participants with pairs of faces (each image presented sequentially for 750ms) which differed only in colour (lightness, redness or yellowness) ranging from a difference of 1.2-9.6 CIE units. They found that regardless of colour change dimension (red, yellow or luminance) or of ethnicity of facial stimuli (Caucasian, Asian or African); raters were reliably able to discriminate colour differences at all levels. The smallest colour difference value found to be perceptible (1.2 units) is less than the colour differences reported in supplementation studies of all skin types (Coetzee & Perrett, 2014; Stephen et al., 2011; Tan et al., 2015), suggesting that, in theory, these colour differences, attributable to carotenoids, are sufficient to be perceived. Further perceptual work suggest that the magnitude of colour change required to influence judgements of health, is larger than that required to be perceivable (Re et al., 2011; Whitehead, Re, et al., 2012). When considering a colour change associated with blood perfusion and oxygenation, Re et al (2012) found that a difference as small as 0.6 units was sufficient to be perceivable but a difference of 1.44 units was necessary to influence judgements of health. Similar values were reported by Whitehead et al (2012) in their investigation of carotenoid colour (a 0.89 unit difference was perceivable and 1.37 was necessary to influence judgements of health). Both of these studies however were limited to Caucasian stimuli. Assuming that these thresholds are directly applicable to other skin types, the colour change reported in African skin with carotenoid supplementation could be deemed great enough to influence judgements of health.

In summary then, across several studies it had been demonstrated that skin yellowness or carotenoid colouration of human skin is judged to be attractive and healthy looking, in a range of skin types. Secondly, a range of skin types show some change in skin yellowness with carotenoid supplementation, although in deeply pigmented African skin this has only been demonstrated at the palm. Finally, these colour changes, measured in response to carotenoid supplementation are sufficiently large to influence judgements of health *in theory*. Together this body of literature provides compelling evidence that carotenoid colouration of skin could be used to inform judgements of health across a range of skin tones. What the current literature lacks at present however, is a single, self-contained demonstration that this is true.

Here, in Study 1a, 10 individuals who varied in ethnic background and constitutive skin tone, took part in a carotenoid supplementation study. They were provided with two extra

vegetable portions per day (one pepper and 250ml of carrot juice) with which to supplement their diet over a six week period; skin colour was assessed during this time. In Study 1b, we used photographs of these same participants at baseline and after 6 weeks of supplementation to assess the effect of carotenoid supplementation on apparent health. The perceptual arm of this investigation was conducted under two conditions, photographs were shown either raw and un-manipulated or with a personalised colour transformation applied to the baseline photograph which reflected only the skin colour change recorded. Unlike prior perceptual work, the carotenoid transform which was applied was personalised, reflecting only the colour change recorded in that individual, not the average colour change recorded across the group. Both conditions were important because each have their own strengths and limitations. Raw photographs provided maximum ecological validity, but could be confounded by other changes in appearance (e.g. expression and general grooming). Participants in the supplementation study were not blind to the hypothesis and could have anticipated looking more attractive. Colour only transforms control for all other variation in appearance, but take a colour change which is measured in a single location and apply it uniformly across the face. This may not reflect the distribution of carotenoid colouration in facial skin. In both Studies 1a and 1b, comparative analysis is also conducted according to a median split by baseline skin lightness to test whether change in skin colour or associated health judgements vary in lighter and darker skin tones.

2.3. Study 1a: Carotenoid supplementation

2.3.1. Methods

Design

A repeated measures experimental design was employed to test the effects of increased carotenoid consumption on skin colour.

The study arose opportunistically as the result of a public engagement opportunity with television broadcaster the BBC. The BBC requested a demonstration of the skin colour effects associated with carotenoid consumption as reported by Whitehead and colleagues (2012). Constraints around sample size, participant ethnicity, time period of data collection, and choice of vegetables stemmed directly from the collaboration. Nevertheless data collected during this project provided an opportunity to empirically measure skin colour change in a sample of mixed ethnicity participants during increased carotenoid consumption.

Participants

10 participants (4 male, 6 female, mean age 28, SD 8.31) with a diverse range of constitutive skin pigmentation took part in the experiment. Participants self-reported ethnicity in the following manner: Caucasian *n*=5, Indian *n*=2, Chinese *n*=1, Afro-Caribbean *n*=1, Mixed *n*=1. It should be noted that ethnicity is a subjective term and we found that at times over the course of the study the same individuals used different terms to classify their own ethnic background.

Procedure

Participants received 1 pepper and 250ml of carrot juice per day over 6 weeks which they were asked to supplement their diet with. The choice of vegetables provided was a constraint related to the collaboration. The dosage was chosen to be consistent with 2 extra portions of vegetables per day which was suggested by Whitehead et al (2012) to be sufficient to cause a measurable change in skin colour. Participants were asked not to replace any fruits or vegetables they would normally eat with those provided but to consume them in addition to their normal fruit and vegetable intake.

Participants' skin colour was measured at baseline, and at weeks 1, 3, 4, 5 and 6. Skin measurements were taken spectrophotometrically at 6 body locations: palm, inner forearm, tricep, shoulder, cheek and forehead which were averaged to produce global skin colour measurements in terms of lightness (L*), redness (a*) and yellowness (b*) at each time point.

Additionally, facial photographs were taken of all participants at baseline and after 6 weeks of carotenoid supplementation for use in Study 1b.

Compliance

Each week, participants were asked whether they had consumed all provided vegetables the previous week and if not, how many portions had been missed. Six participants reported full compliance across the duration of the experiment. Two participants reported missing 1 portion each in either week 2 or week 6. Two participants missed 4 portions each in either week 3 or week 4 and 1 participant reported missing a total of 6 portions over the course of the study (4 in week 3 and 2 in week 6). Of the 840 portions of vegetables provided to participants, only 12 (1.43%) were not consumed.

Statistical Analysis

Repeated ANOVAs were used to assess how skin lightness, redness and yellowness varied over time in response to carotenoid supplementation. Where Mauchly's test of sphericity revealed a violation of this assumption, a Greenhouse-Geisser correction was applied. Significant colour changes were further explored by body location and constitutive skin tone, again using repeated ANOVAs

For comparative analysis of skin tone, subjects were divided according to a median split with regard to skin lightness values at baseline. All participants in the high lightness (n=5, Mean 66.45, SD 1.72) group reported a Caucasian ethnicity and all individuals in the lower lightness group (n=5, Mean 56.88 SD 8.77) reported a mix of non-Caucasian ethnic backgrounds (Indian n=2, Chinese n=1, Afro-Caribbean n=1, Mixed n=1). Images showing average baseline skin colour of the participants in each group are presented in Study 1b.

Three out of 10 individuals were unable to attend colour measurements at the three week mark. To save statistical power, and make all comparisons valid (i.e. containing all participants), this time point was excluded from analysis.

2.3.2. Results

Global Colour

A two-way repeated ANOVA between colour (lightness, redness and yellowness) and time (baseline, week1, week4, week5, week6) showed a significant interaction. $F(8,72)=5.40$, $p<.001$, $\eta^2_p = .375$. For the yellowness dimension, a repeated ANOVA confirmed a significant effect of time ($F(1.87,16.83)=13.01$, $p<.001$, $\eta^2_p= .591$) best described as a linear trend $F(1,9)=18.27$, $p=.002$, $\eta^2_p =.670$). Bonferroni[1] corrected pairwise comparisons confirmed a significant difference from baseline at weeks 4 ($p=.023$), 5 ($p=.020$) and 6 ($p=.034$).

Skin redness did not vary significantly by week ($F(4,36)=1.78$, $p=.155$, $\eta^2_p = .165$); nor did skin lightness ($F(4,36)=.13$, $p=.921$, $\eta^2_p = .025$). See Figure 2 for a description of skin colour change over time.

[1] Bonferroni corrections were applied by SPSS and p values adjusted appropriately so that $p<0.05$ is still considered statistically significant at an alpha level of 0.95.

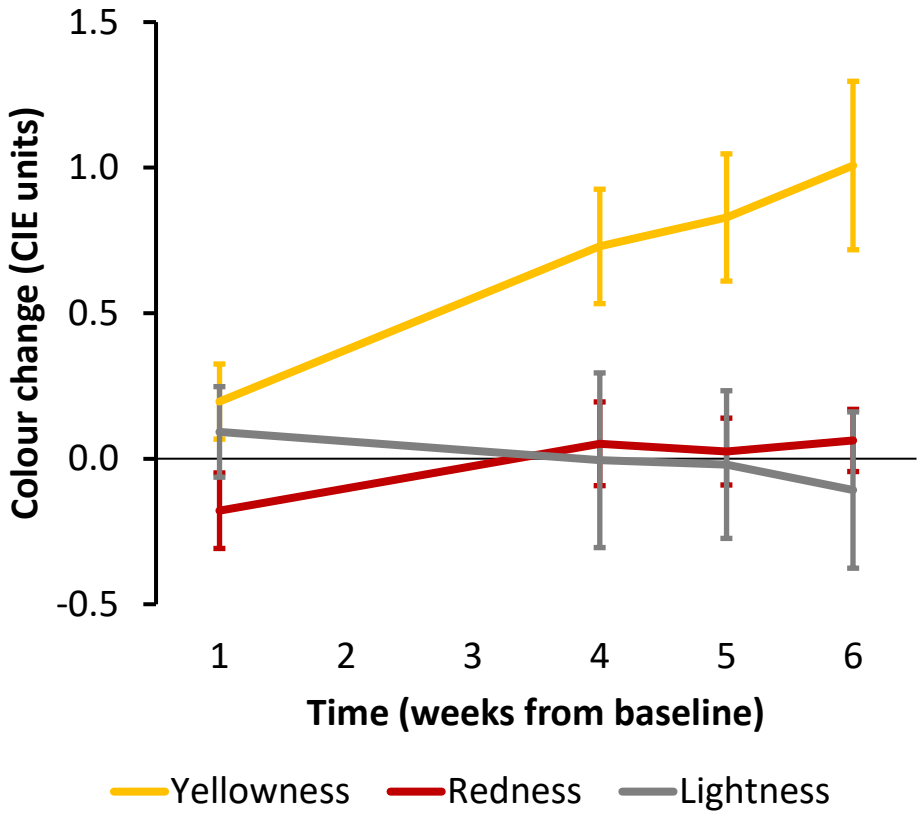

Figure 2: Global colour change over time in response to two extra portions of vegetables per day in a sample of 10 participants. Skin yellowness increased steadily over time whilst redness and lightness remained constant. Data points represent group level change from baseline. Error bars represent 95% confidence intervals around change from baseline.

The ANOVA for skin yellowness over time was repeated with the inclusion of the between subjects binary variable constitutive pigmentation (higher and lower skin lightness). The main effect of time remained ($F(1.79,14.28)=12.36$, $p<.001$, $\eta^2_p= .607$). There was also a main effect of pigmentation. Individuals with darker skin tones, had more yellow skin (Mean 18.89, SD 2.21) than individuals with lighter skin (Mean 15.58, SD 1.35) ($F(1,8)=796.66$, $p<.001$, $\eta^2_p= .990$) but there was no interaction between pigmentation and week ($F(1.79,14.28)=.547$, $p=.571$, $\eta^2_p= .06$). Figure 3 shows that the change in yellowness over time was of similar magnitude amongst both sub-groups (i.e. although light skin toned individuals showed a higher increase in yellowness, error bars overlap at all time points demonstrating that there was no statistically significant difference).

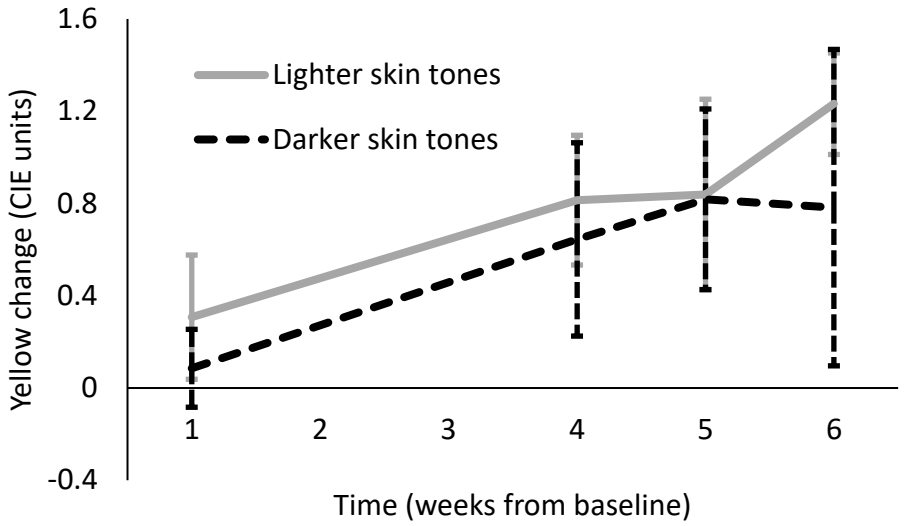

Figure 3: Change in skin yellowness amongst groups with lighter (solid line, *n*=5) and dark (dashed lines, *n*=5) constitutive skin tones. Both groups show an increase over time in response to 2 extra portions of vegetables per week. Error bars represent 95% confidence intervals around change from baseline.

Yellowness by Location

A repeated ANOVA comparing skin yellowness before and after supplementation (week 0 and week 6) and at all locations (palm, inner forearm, tricep, shoulder, cheek and forehead) showed a significant main effect of time ($F(1,9)=15.50$, $p=.003$, $\eta^2_p = .633$) but no main effect of location ($F(5,45)=.73$, $p=.61$, $\eta^2_p = .075$) and only a trend for an interaction between time and location ($F(5,45)=2.28$, $p=.063$, $\eta^2_p = .20$). This confirms that skin yellowness increased across all participants between week 0 and week 6 but there was no significant difference in the degree to which body locations increased in yellowness. Figure 4 shows change in yellowness by location. The bar graph shows that change was highest is the palm and forehead and lowest at the tricep and shoulder, although these differences were not significant.

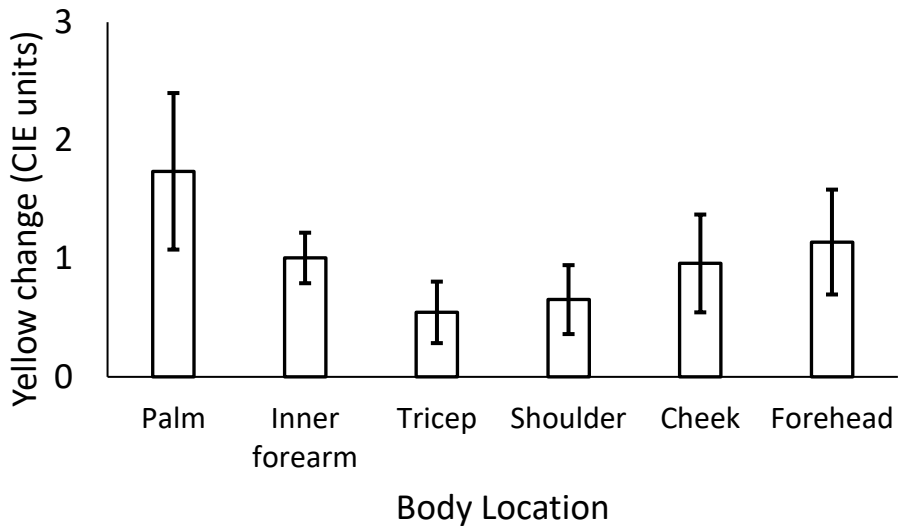

Figure 4: Colour change by location in response to a dietary increase of two vegetable portions per day over six weeks.

2.4. Study 1b: Health perception

2.4.1 Methods

Design

A forced choice experimental design was employed to test the effects of carotenoid supplementation on perceived health. Before and after images were presented in two within subject conditions: raw images and colour only transforms

Materials

Photographs were taken of participants in Study 1a at baseline and after six weeks of carotenoid supplementation, with a Fujifilm FinePix S5Pro digital SLR camera. Participants sat a standardised distance from the camera, and adjusted the height of their chair so that their eyes were level with the camera lens. Clothing was covered with a whiteboard to prevent colour spill, all jewellery was removed. Participants were asked to maintain a neutral expression.

In *psychomorph* (freely available online software for manipulating facial images), photographs were cropped and aligned; patches of skin from the forehead of each image were then colour analysed. For each pair of images, the colour difference between baseline and week 6 was calculated and then applied to a copy of the baseline images. This resulted in a

set of “after” images showing colour only change whilst keeping constant all other features such as hairstyle, skin texture and expression.

Participants and procedure

136 participants took part in an online forced choice experiment. In a 2X2 design, participants were presented with identity matched photos of individuals before and after six weeks of carotenoid supplementation. Each identity was shown twice; either as raw images or images altered to show colour change only. On each trial participants were asked to “Please click on the face that looks healthier”

Statistical Analysis

The proportion of trials on which the after image was selected as healthier was calculated for each participant and separately for each condition.

One sample t-tests were used to compare results to chance (50%). Residuals were found to be normally distributed.

A repeated 2x2 ANOVA was employed to compare results across trials by condition (raw images versus colour only transforms) and constitutive pigmentation of stimuli (lighter and darker skin tone). Where Mauchly’s test of sphericity revealed a violation of this assumption, a Greenhouse-Geisser correction was applied.

2.4.2 Results

Averaged images

To illustrate the effect of the supplementation on facial appearance, average before and after images were created in *psychomorph*. This was conducted separately for individuals with

lighter (n=5) and darker (n=5) skin tones. Images can be viewed in Figure 5.

Figure 5: Average facial images before (left) and after (right) carotenoid intervention separated into individuals with the highest (top) and lowest (bottom) baseline skin lightness values. Each composite is made from 5 individual images. Composites are used to illustrate typical faces of each category. Faces used in the ratings tasks were photographs of real individuals.

Overall

On average, participants selected the after image as healthier on 6 out of 10 trials for both raw images (mean= .63, SD= 0.16) and colour only transforms (mean= .62, SD= 0.18).

One-sample t-tests confirmed that this preference was significantly above chance both for raw images ($t(135)=9.61$, $p<.001$) and colour only transforms ($t(135)=7.80$, $p<.001$).

By constitutive skin tone

On average, participants selected the after image as healthier on 3 out of 5 trials for both raw images and colour only transforms and with respect to both groups of skin tones (see Figure 6).

One-sample t-tests confirmed that in all conditions, the preference for carotenoid supplemented images was significantly above chance (all *p*s>.001).

A repeated measures ANOVA confirmed no main effect of skin tone ($F(1,135)=1.04$, $p=.309$, $\eta^2_p = .008$) or condition ($F(1,135)=.152$, $p=.697$, $\eta^2_p = .001$) but did show a significant interaction ($F(1,135)=6.79$, $p=.010$, $\eta^2_p = .048$). For stimuli with lighter skin tones, the preference for post supplementation images was stronger when colour was isolated, whereas amongst darker skin tones, the preference was stronger with raw images.

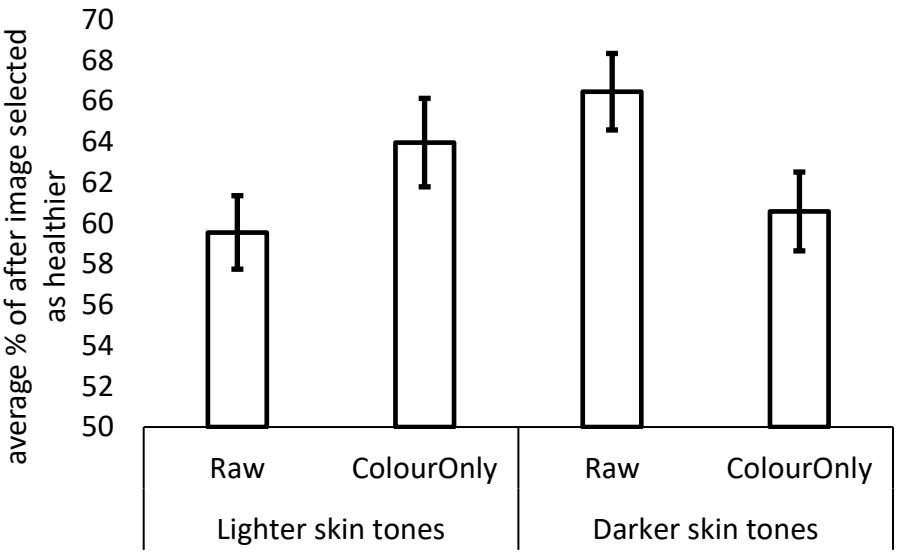

Figure 6: Average % of after images (out of 5) selected as most healthy in a comparison of before and after carotenoid intervention. Results shown by constitutive pigmentation of stimuli and image comparison (raw, un-manipulated images versus those with colour difference only). Error bars represent 95% confidence intervals around the mean.

2.5. Discussion

Results of the two presented studies demonstrate that in a sample of individuals with varied skin tones, a modest increase in dietary consumption of vegetables (two portions per day) led to a measurable change in skin yellowness. Furthermore, this skin colour change was shown to favourable influence judgements of apparent health.

The study was conducted during winter months (December-January) and so the increase in skin yellowness cannot be explained by an increase in sun exposure. The increase in skin yellowness occurred as predicted and is consistent with prior supplementation studies (Alaluf, Heinrich, et al., 2002; Coetzee & Perrett, 2014; Stahl et al., 1998; Stephen et al., 2011; Tan et al., 2015) but adds to prior work by demonstrating that a naturally obtained change in skin colour with fruit and vegetable consumption can influence judgements of health. Two prior studies investigating fruit and vegetable consumption and skin colour have shown an increase in redness as well as yellowness with carotenoid supplementation (Tan et al., 2015; Whitehead, Re, et al., 2012), although the change in redness was less prominent than that of the yellowness. This could have occurred for one of two reasons, either because lycopene (the most common carotenoid in human skin) is more red in appearance than other carotenoids; or

because fruit and vegetable consumption improves blood circulation in upper layers of the skin (De Spirt, Sies, Tronnier, & Heinrich, 2011). Findings from the present study which recorded no change in redness, give support to the former explanation, and suggest that a diet rich in lycopene could increase redness as well as yellowness.

Results showed that change in yellowness occurred as early as 4 weeks post supplementation which is in line with findings from Tan et al. (2015). We also found there was no difference in the magnitude of change in yellowness amongst individuals with the lightest and darkest constitutive skin tones within our sample. This later finding is somewhat surprising as we may expect the effect of carotenoid deposition to be less visible in darker skin; and a limitation of the present study was that two individuals in our darker group were relatively light in skin tone, with values close to those in the lighter group. Findings are however consistent with evidence from 2 carotenoids supplementation studies which found a similar magnitude of change in yellowness of African (1.67 CIE units) and Caucasian (1.26 CIE units) palm skin after supplementation with carotenoid capsules ((Coetzee & Perrett, 2014) and (Stephen et al., 2011) respectively), although it should be noted that no colour change was noted at other measured locations in African skin.

With regard to perceptions of health, again, there was no main effect of constitutive skin tone on preference for the post supplementation skin colour. In groups of both lighter and darker skin tones, after supplementation images were selected as more healthy on average 60% of the time (3 out of 5 trials) and at a level significantly above chance. Perceptions of health in response to carotenoid supplementation were tested across two conditions. Whether images were shown as raw, un-manipulated photographs or with colour only transformations, raters selected the after image as more healthy on an average of 6 out of 10 trails, again this was significantly above chance and suggests that the finding is robust. Raw images have the advantage of accurately portraying the distribution of skin colour changes and are therefore more ecologically valid. They do however suffer the limitation that other systematic difference in appearance could influence judgements (hair style or expression for example, where difference may arise as expectancy effects). Colour only transformations on the other hand suffer the limitation that colour change is measured in a specific location (the forehead) and applied globally across facial skin which may not reflect the distribution in colour change which actually occurred. One strength of the current study is that when the colour transformation was applied, this was personalised for each pair of faces, so the colour difference represented the colour change relevant to that particular individual and not the

average colour change that occurs with carotenoids supplementation as has been used in prior perceptual studies (Stephen et al., 2011; Whitehead, Coetzee, et al., 2012; Whitehead, Re, et al., 2012). This insures that the colour change is relevant to the skin tone to which it is applied. Regardless of the way in which images were presented though, across all stimuli, preference for after colour was unaffected. This would suggest that even when other information is available, raters are paying attention to and making decisions based upon skin yellowness, if they were not, we would expect preference to be stronger in colour only trials where the most relevant cue is isolated.

There was evidence of an interaction between presentation of stimuli and constitutive skin colour of stimuli. For stimuli with lighter skin tones, preferences for post supplementation images was higher when colour was isolated, whereas amongst stimuli with darker skin tones, performance was higher with raw images. The effect size of this interaction was small, and could have been driven by individuals with darker skin tones who were slightly more smiley in their post supplementation photographs, this effect can be seen in the composite images. The important take home finding is that preference for images following an increase in fruit and vegetable consumption was significantly above chance in groups of individuals with both lighter and darker skin tones and irrespective of stimulus presentation.

A limitation of the present study is the small sample size of participants in Study 1a. The effects of both colour change and perceived health were highly significant and therefore reliable but our sample of individuals with darker skin tones contained only 1 participant reporting an Afro-Caribbean ethnicity and showing very high levels of melanin pigmentation (i.e. low lightness values). Despite this limitation, the difference in pigmentation between groups of individuals with lighter and darker skin tones was large (as evident from composite images and L* values). This difference provides reasonable grounds to believe lack of difference in colour change with carotenoid supplementation between these groups is meaningful and carotenoids are likely to be as influential in darker skin tones as in lighter skin. Nevertheless, a larger sample of participants varying in constitutive skin pigmentation would provide the power for a more nuanced correlational assessment of the relationship between constitutive skin pigmentation and visibility of skin carotenoids.

Carotenoid intake was not strictly controlled in this study. Measured compliance showed that participants consumed almost all of the provided vegetables (96%), however, it is possible that participants ate additional fruits and vegetables in excess of those provided. Indeed,

during debrief, some participants spoke of the study providing extra motivation to eat healthily. We can therefore not conclude equivocally that 2 portions are sufficient to improve appearance but can conclude that an achievable change in carotenoid intake can increase judgements of apparent health.

A final limitation is the forced choice presentation of stimuli in the perceptual study. This design was selected in order to isolate the effect which we were testing (carotenoid colouration of skin) but arguably does not represent the manner in which judgements of health would be made in the real world. Future work could use a single presentation rating study to assess perceived health in a manner that would be considered more ecologically valid.

In summary, whilst prior work has provided strong evidence that fruit and vegetable consumption can visibly influence skin colouration in a manner and magnitude that *in theory* should affect judgements of health; this study provides the first self-contained demonstration that an achievable increase in vegetable consumption *does* favourably influence judgements of health. Furthermore this was found to be true in individuals of both lighter and darker skin tones within the sample. With an increase in only 2 portions of vegetables per day a measurable change in skin yellowness was recorded within 4 weeks and photographs taken after 6 weeks demonstrated that the effect was favourable. These findings support the hypothesis that natural variation in skin carotenoid levels can act as an informative cue to health status in individuals of varying skin pigmentation.

Chapter 3: Carotenoid colour and risk factors for health outcomes

3.1. Chapter outline

This chapter explores the relationship between skin yellowness and various risk factors related to health under the premise that carotenoid ornaments in non-primate species are believed to signal a global property of condition. Smoking, alcohol consumption, lack of exercise, excess body weight and psychological stress are all well known risk factors to health and presumably would compromise a global index of condition. Across 2 studies these risk factors were investigated, along with fruit and vegetable consumption, in relation to carotenoid colouration of human skin. Study 3 investigates these factors across a sample of young Caucasian participants, whilst Study 4 investigates how changes in these factors relate to changes in skin colour within individuals. Both studies show a significant effect of psychological stress, with high levels related to low carotenoid colouration and an increase in stress within participants linked to a reduction in skin carotenoid colour. The link between fruit and vegetable skin colour is confirmed in Study 3 but not Study 4. All other factors show no significant relation to carotenoid colour of skin. Results demonstrate that carotenoid colour of human skin reflects health relevant information beyond diet (i.e. stress) yet many of the predations were unsupported. Results are discussed in light of the limitation that participants were predominantly young and healthy and that current condition may not be best represented by the factors measured.

3.2. Introduction

Carotenoid signals in birds are thought to be a condition dependent trait. Individual "condition" of an animal has been defined by (Hill, 2011) as an emergent characteristic capturing variation in somatic state, genotype and epigenetic state. In other words, colouration is global index encompassing an individual's current state of health, propensity to resist ill-health and ability to recover from challenges. This may be mediated by physiological pathways including immune function and oxidative stress (see Chapter 1.2)

In humans, a number of individual differences and lifestyle behaviours are known risk factors for health outcomes. Obesity, diet, smoking, lack of exercise, psychological stress and excess alcohol consumption all have well established links to risk of specific diseases and all-cause mortality. Presumably, if condition could be measured directly, these risk factors would affect it. Further, if carotenoid colouration of human skin reflects condition we may expect these risk factors to be reflected in carotenoid colouration of human skin.

Below, evidence of the links between health outcomes and selected risk factors is summarised. In some instances specific physiological pathways are highlighted thorough which carotenoid colouration of skin may be affected (e.g. oxidative stress or immune function). An empirical investigation of the effects of these health risk factors upon carotenoid colouration of skin follows with two studies. The first tests how variation in diet, stress, exercise, and obesity relates to variation in skin yellowness across Caucasian individuals. The second study tests how skin yellowness changes in response to changing levels of exercise, fruit and vegetable consumption, stress and body fat.

3.2.1. Smoking

Health risks associated with smoking have been well known for the last half a century. In 1964, a landmark report from the Surgeon General of the United States was published which became the first in a series of many reports linking smoking to several diseases. Primarily, smoking was established as a major risk factor for lung cancer, chronic bronchitis, heart disease, and atherosclerosis. Later, the 2004 Surgeon General's report showed that every organ of the human body is affected by smoking (U S Department of Health and Human Services, 2014).

Looking specifically at the links between smoking and carotenoids, there is evidence from a large scale observational study (*n*=849) that current smokers had lower levels of carotenoids alpha and beta carotene relative to non-smokers despite no difference in intake of high carotenoid vegetables (Aoki, Ito, Sasaki, Ohtani, & Hamajima, 1987). The authors of this study also found that heavier smokers had lower beta-carotene levels than light smokers but only amongst those who also drank alcohol. In another, larger and more recent observational study of European individuals (*n*=3043) smoking status was again found to predict plasma carotenoid levels. The effect was smaller than that of region, gender (with females showing higher levels) and BMI (Body Mass Index: a measure of weight relative to height), but larger than that of alcohol intake (Al-Delaimy et al., 2004). Plasma carotenoid levels (of lutein, zeaxanthin, lycopene and beta-carotene) have also been shown to increase four weeks after smoking cessation (Polidori, Mecocci, Stahl, & Sies, 2007).

The reduction of plasma carotenoid levels associated with smoking is likely caused by an increase in oxidative stress. Cigarette smoke contains free radicals (Church & Pryor, 1985) and smokers have been shown to have higher levels of DNA-repair product 8-hydroxydeoxy-guanosine (8-OH-dG) in their urine which is a biomarker of DNA damage from oxidative stress (Loft et al., 1992). Indeed, Polidori and colleages (2007) found that with smoking cessation, in addition to an increase in plasma carotenoid levels, other markers of oxidative stress and ability to deal with oxidative stress were improved. Conversely, one large scale epidemiological study (n=1797) of adults in New York found no evidence of smoking status on oxidative stress but suggest this may be due to passive smoking which could be an important unmeasured confounder (Trevisan et al., 2001). A review paper of over 100 experimental studies investigating the effects of acute cigarette smoke exposure upon inflammation and oxidative stress identified twelve studies specifically measuring markers of oxidative stress. All twelve of these studies found an increase in oxidative stress following exposure to cigarette smoke, suggesting that smoke exposure and not only smoker status could be responsible for increased oxidative stress (van der Vaart, Postma, Timens, & ten Hacken, 2004).

3.2.2. Alcohol

Alcohol consumption is causally related to more than 60 different medical conditions according to a review by Room et al., (2005), including cancers, diabetes, cardiovascular

disease, and gastrointestinal disease. The relationship between alcohol and health outcomes is a complex one though. Whilst some diseases, for example breast cancer, were found to show a linear relationship, others are related to a pattern of irregular and heavy drinking (e.g. stroke or sudden cardiac death). Results of a comprehensive meta-analysis also showed low-to-moderate consumption (less than two drinks per day) predicts the lowest risk of coronary heart disease, with the overall relationship between coronary heart disease and average volume of alcohol consumption showing a J-shaped curve (Corrao, Rubbiati, Bagnardi, Zambon, & Poikolainen, 2000).

In relation to carotenoid levels, the two large scale observational studies described above in relation to smoking and plasma carotenoid levels, both also found significant effects of alcohol consumption. Aoki et al., (1987) found that individuals who reported drinking more than three days per week had lower plasma carotenoid levels, particularly beta-carotene, relative to non-drinkers (occasional drinkers were excluded from analysis). In the second and larger study, Al-Delaimy and colleagues also found a significant effect of alcohol intake upon plasma carotenoids amongst their 3043 participants (Al-Delaimy et al., 2004). This time a linear effect was reported with higher alcohol intake predicting lower plasma carotenoids although the effect size was small, explaining only 2% of variance in plasma carotenoid levels. Another study found no link between alcohol consumption and plasma carotenoid levels in their similar epidemiological survey of 1797 adults (Trevisan et al., 2001). In explaining the findings the authors point to the fact that some alcoholic beverages will contain antioxidants and that interactions between alcohol consumption and dietary antioxidants are largely unknown. In support of this interpretation, it has been shown that alcohol from beer and liquor but not from wine is significantly and positively associated with at least one marker of oxidative stress (thiobarbituric acid-reactive substances) (Kannel & Ellison, 1996).

Another experimental study of the effects of alcohol upon oxidative stress found that when alcohol was given to human participants in incremental doses served as alcohol solution made up to 240ml with lemonade, urinary isoprostanes (a product of free radical attack of essential fatty acids and a biomarker of oxidative stress) increased in a time and dose dependant manner to the alcohol content (Meagher et al, 1999). However, when compared to a placebo group given lemonade, only the two largest doses (0.6, or 0.9g of 98% solution of alcohol per kg body weight) were associated with a significant increase in urinary

isoprostanes. This particular experimental study suggests then that small amounts of alcohol may not be harmful in terms of oxidative stress.

3.2.3. Psychological stress

A classic study by Cohen, Tyrrell, and Smith (1991) showed that individuals who reported higher levels of psychological stress were more likely to develop the common cold after exposure to respiratory viruses through nasal drops. Subsequently, a meta-analysis of stress and immunity studies has concluded that stress reduces both the number of immune cells and the ability of immune cells to perform specific activities (Herbert & Cohen, 1993). Suppression of the immune system may also mediate the effects of stress on wound healing. A systematic review and meta-analysis found that stress (defined as any form or negative psychological state, condition or experience) was associated with impaired healing or deregulation of biomarkers related to wound healing (Walburn, Vedhara, Hankins, Rixon, & Weinman, 2009). Psychological stress, particularly prolonged stress has also been linked to increased risk of cardiovascular disease (Dimsdale, 2008). Stress may also contribute to the initiation, growth and metastasis of tumours (Cohen, Janicki-Deverts, & Miller, 2007).

In addition to suppression of the immune system and increased risk of some chronic diseases, there is also evidence that psychological stress is related to lower plasma levels of carotenoids and elevated markers of oxidative stress. One study comparing nurses in high stress jobs to those in low stress jobs (confirmed with the Brief Job Stress Questionnaire) found that those in higher stress jobs had lower levels of plasma beta-carotene (Tsuboi et al., 2006). Exam stress, too is associated with markers of high oxidative stress including reduced plasma antioxidant capacity and an increased sensitivity of lipids and DNA to oxidative damage noted the day before an exam relative to mid semester (Sivonová et al., 2004). Similarly, levels of bilirubin oxidative metabolite (BOM) in the urine of a group of individuals who had delivered a talk at a conference were found to be higher than a comparison group who attended but did not give talks (Yamaguchia, Shiojib, Sugimotoc, & Yamaokad, 2002). Bilirubin is a breakdown product of haemoglobin, often thought to be a toxic and waste product, but is able to act as a powerful antioxidant providing a strong protective function when the mechanisms of defence against oxidative stress are challenged (Yamaguchia et al., 2002). Presence of BOMs suggests that bilirubin has been expended in defence against reactive oxygen species. Self-reported ratings of perceived psychological

stress confirmed that the group giving talks felt more stressed than those attending only. Furthermore, amongst those giving talks, BOM levels were found to be linearly related to self-reported stress.

Chronic psychological stress has also been linked to elevated oxidative stress. Allostatic load is a term that refers to the physiological wear and tear of the body in response to chronic stress (McEwen, 1998); and in a large scale observational study of American adults (*n*=3387) it has also been found that individuals with higher measures of allostatic load have lower levels of serum beta-carotene (Rosenberg, Park, & Eldeirawi, 2014). In this particular study allostatic load was calculated based on nine physiological indicators (systolic and diastolic blood pressure, pulse rate, total and HDL-cholesterol, glycosylated Hb, sex-specific waist-to-hip ratio, albumin and C-reactive protein). The relationship between serum beta-carotene levels and allostatic load remained after controlling for age, education, race/ethnicity, smoking, alcohol consumption and physical activity.

3.2.4. Exercise

Regular physical activity is known to be beneficial in the prevention of a host of chronic diseases (e.g., cardiovascular disease, diabetes, cancer, hypertension, obesity, and osteoporosis) as well as all-cause mortality (Warburton, Nicol, & Bredin, 2006). Interestingly recent evidence from a cross-sectional observation study also suggest that regular exercise may have protective effects against susceptibility to the common cold (Nieman, Henson, Austin, & Sha, 2011). Nieman and colleagues tracked over 1000 participants for a period of 12 weeks during autumn and winter. They found that exercise frequency was linearly related to the reported number of days which individuals experienced symptoms of upper respiratory tract infections and the severity of these symptoms (as measured by Wisconsin Upper Respiratory Symptom Survey). Participants in this study who exercised more frequently also had lower BMI scores and lower stress levels but the effect of exercise on symptoms of respiratory infections was independent of these variables.

A systematic review of 19 studies investigating the effects of sedentary behaviours on health outcomes among adults concluded that there is strong evidence that sedentary behaviour is related to all-cause mortality and mortality resulting from cardiovascular disease (Proper, Singh, Van Mechelen, & Chinapaw, 2011). A larger review of 48 longitudinal studies between 1996 and 2011 investigating the effect of sedentary behaviour on health outcomes in

adults concluded that sedentary behaviour may be a distinct risk factor, independent of physical activity in predicting health outcomes. Results suggested that sedentary behaviour predicts mortality and weight gain from childhood but findings were mixed in relation to cardiometabolic risk and insulin resistance (Thorp, Owen, Neuhaus, & Dunstan, 2011). Whilst there is a wealth of evidence that average physical activity levels are inversely related to a host of outcomes, it is unclear whether long periods of sedentary behaviour is an addition and independent risk factor.

There are limited and mixed findings upon the effects of exercise (acute or regular) upon carotenoid levels. Vierck and colleagues measured skin carotenoids using Raman spectroscopy following bouts of endurance exercise (in the form of cycling and running). They report that carotenoid concentrations measured at the palm and forehead were reduced in response to both activities (Vierck et al., 2012).In terms of regular exercise, an investigation on the effects of cycling upon antioxidant levels in cyclists found no difference in plasma carotenoids between amateur and professional cyclists (Aguiló et al., 2003). The same study found that amateur cyclists showed no change in plasma carotenoids (or other measure antioxidants) following a sub maximal or maximal exercise test but conversely, professional cyclists did show a drop in a plasma carotenoids following a session of endurance exercise. In another study, plasma antioxidant levels (including beta-carotene) were measured in separate samples of young and elderly individuals who exercised regularly (minimum 3 session per week, each >1hour) or did not. Results showed that amongst young participants, those who exercised regularly had higher plasma carotenoids levels, but amongst elderly participants, there was no difference in carotenoid levels amongst those who exercised and those who didn't. Elderly participants who exercised had lower plasma carotenoid levels than young participants who exercised (Rousseau, Margaritis, Arnaud, Faure, & Roussel, 2006). It appears that exercise may then have protective benefits amongst young individuals but the authors suggest exercise cannot counteract the increased oxidative stress associated with ageing. No epidemiological studies (or small scale investigations) were identified which tested the relationship between frequency of exercise and plasma carotenoid levels at a population level.

In terms of oxidative stress, it is generally thought that whilst acute or endurance exercise may increase oxidative stress, this leads to important physiological adaptations that ultimately upregulate the body's endogenous antioxidant defences and therefore increases ability to deal with future episodes of oxidative stress (Fisher-Wellman & Bloomer, 2009; Gomes, Silva, &

Oliveira, 2012). This is consistent with evidence demonstrating that the threshold for exercise induced oxidative stress is related to training status of an individual (Møller et al., 1996), and may help to explain some of the mixed findings around changing carotenoid levels with exercise. The potential long term benefit of regular exercise upon skin carotenoids and skin colouration is not yet tested.

3.2.5. Adiposity

Obesity (generally classified as a body mass index (BMI) greater than 30Kg/m) is a well-established risk factor for numerous chronic diseases including cardiovascular disease, diabetes, obstructive sleep apnoea, and cancers, most of which are thought to be triggered through a pathway of chronic inflammation and oxidative stress (Dixon, 2010).

BMI is also inversely related to plasma carotenoids (Burrows et al., 2015). Indeed, BMI was found to be a stronger predictor of plasma carotenoid levels than smoking status and alcohol consumption amongst more than 3000 participants (Al-Delaimy et al., 2004). Again, in a sample of 285 healthy adolescents, obesity was found to be inversely related to serum carotenoids, whilst controlling for intake of fruits and vegetables (Neuhouser et al., 2001). In children too (*n*= 93 aged 5-12), overweight and obese individuals have lower levels of carotenoids: beta-carotene, alpha-carotene and crytoxanthin, independent of measured carotenoid intake (Burrows, Warren, Colyvas, Garg, & Collins, 2009). In a smaller sample of 100 participants, total body fat and was inversely related to retinal levels of carotenoids (lutein and zeaxanthin) but not to serum levels in both men and women (Bovier, Lewis, & Hammond, 2013). Hammond and colleagues also found an inverse relationship between body fat and the measured density of macular (a small area of the retina) carotenoid pigments which is proportional to levels of lutein and zeaxanthin. (Hammond, Ciulla, & Snodderly, 2002).

Carotenoids are fat soluble, and so one potential explanation for the inverse relationship between BMI and plasma carotenoid levels is that they are being locked away in stores of fat. This explanation is unlikely however, as high levels of body fat have been shown to be inversely related to measured carotenoids in adipose tissue (Chung et al., 2009). An alternative explanation is that accumulation of adipose tissue is a direct and independent contributor to oxidative stress. In support of this explanation, several measures of obesity have been shown to correlate with markers of oxidative stress in population studies, including

body mass index (BMI), waist circumference, and body fat weight (Vincent, Innes, & Vincent, 2007). Furthermore, one study in mice has specifically found that white adipose tissue, but not other tissue of obese mice, was associated with increased free radical production and decreased expression of antioxidant enzymes, relative to controls (Furukawa et al., 2004). They also found that in cultured adipocytes (fat cells), elevated levels of fatty acids increased free radicals; and finally that in obese humans, BMI and waist size correlated positively with systemic levels of oxidative stress.

Higher BMI has been associated with lower skin carotenoid levels at the palm as measured by Raman spectroscopy (Lima & Kimball, 2011) but whether variation is sufficient to influence the appearance of human skin colour is unknown.

3.2.6. Summary and aims of experimental work

Risk factors described in the above section all have well-established links to health outcomes, and presumably will influence individual "condition". All have also shown direct relationship to plasma carotenoid levels and or metabolic pathways relevant to carotenoid status. Since we know that carotenoids can influence the colouration of human skin (see Chapter 1.3) the experimental work set out in this chapter aims to test whether risk factors relevant to health are also reflected in the yellow appearance of human skin.

Fruit and vegetable consumption, exercise habits, psychological stress, alcohol consumption, and smoker status were all measured via self-report questionnaire and percentage body fat was measured in order to test the hypothesis that carotenoid colouration (yellowness) of human skin will reflect a range of factors relevant to health. Study 3 tests whether variation in skin yellowness between individuals is predicted by the risk factors to health; Study 4 tests whether change in these risk factors is reflected in a change in skin yellowness within individuals. Both between and within participants, it is hypothesised that alcohol consumption, psychological stress and body fat will be negatively associated with carotenoid colouration of skin whilst fruit and vegetable consumption and regular exercise will be positively related. Smoker status is only tested in Study 3 (as no participants changed in smoker status in Study 4), it is predicted that smokers will have less carotenoid colouration of skin relative to non-smokers.

3.3. Study 3 - Cross-sectional investigation

3.3.1. Methods

3.3.1.1. Design

A cross-sectional observation design was employed to determine lifestyle factors which relate to carotenoid colouration of human skin.

3.3.1.2. Participants

Seventy-seven undergraduate students (54 female, 23 male, mean age, 21, age range 18 – 34) were recruited via the University of St Andrews between December 2013 and March 2014. All participants reported being of Caucasian ethnicity and gave informed written consent.

3.3.1.3. Measured variables

Skin colour.

Participants' skin colour (L* a* and b*) was recorded using a Konica Minolta CM-2600d spectrophotometer. For each participant, skin colour was measured in 6 body locations: palm, inner forearm, tricep, shoulder, cheek, and forehead, this sequence of locations was measured five times. Values were averaged first within each location and then across all locations to provide global values of skin colour for each participant in terms of lightness (L*), redness (a*) and yellowness (b*).

Body Measurements.

A Tanita SC-330 was used to measure body weight and composition including percentage body fat.

Diet.

Fruit and Vegetable consumption was assessed using a seven-category food-frequency questionnaire (see Appendix 2), which has previously been shown to correlate with carotenoid colouration of skin (Whitehead, Re, et al., 2012). Participants were asked to report fruit juice, fruit, vegetable juice, salad, vegetable soup, vegetable portions, and potatoes consumed over the past week. Ten response categories were available, ranging from none per week to more than five per day. Participant's responses were re-coded to represent average fruit and vegetable consumption per day. Potatoes were not included in this calculation and were only included in the questionnaire to differentiate from vegetables.

Exercise.

The Godin Exercise scale (Godin & Shephard, 1997) was used to record participants' typical engagement in physical activity (see Appendix 3). Participants reported the typical number of occasions per week during which they engaged in exercise for at least 15 minutes under each of the following categories: strenuous (heart beats rapidly), moderate (not exhausting) and mild (minimal effort). This questionnaire was selected as it has been widely used in epidemiological literature and has been validated against V02max (a measure of physical fitness) in a sample of heathy young adults (Godin and Shephard, 1985).

Stress.

Recent psychological stress (in the past week) was assessed using the seven item stress subscale (see Appendix 4) of the Depression, Anxiety and Stress Scales (DASS-21) (Henry & Crawford, 2005). This scale was selected due to its brevity, the stress subscale of DASS-21 has been validated against the full version in a non-clinical population of over 1000 participants (Henry and Crawford, 2005).

Alcohol.

Participants reported number of alcoholic beverages consumed in the last week for each of the following categories: beer or cider (pints), wine (250ml glass), spirits (20ml shot glass), and mixed drinks e.g., cocktails, long drinks, alcopops (glasses/bottles). Categories and drinks measures were selected which were thought to be familiar to participants so that accurate estimates could be obtained. Participants' responses were re-coded to reflect average units of alcohol consumed over the past week.

Smoker Status.

Participants were asked "Do you smoke or have you recently (in the last six months) quit smoking?" and were given the following response categories: "No, not at all", "yes, currently", "yes, recently quit" One individual reported recently quitting (within the last six months) and was grouped with non-smokers to create a binary variable consisting of current smokers (n=12) and current non-smokers (n=65).

3.3.1.4. Procedure

On arrival participants were asked to wipe their face in preparation for skin colour measurements. They next reported health behaviours as described above and their body measurements were taken using the TANITIA scale. This was followed by skin colour measurements.

3.3.1.5. Statistical analysis

A backwards linear regression was employed to explore the relationship of lifestyle variables to skin yellowness whilst controlling for lightness (melanin) and gender. A backwards linear regression was selected due to the exploratory nature of the study. Backwards regression maximises power to find true effects among many predictors by reducing degrees of freedom in the final model. Predictor variables included in the model were: stress, fruit and vegetable intake, alcohol consumption, smoker status, Godin exercise score and percentage body fat.

3.3.2. Results

Table 1 shows descriptive values for linear variables and Table 2 shows frequency counts for nominal variables. Zero order correlations between all linear variables can be viewed in Table 3. Zero order correlations remain materially the same when controlling for gender (see Appendix 5).

Table 1: Means and standard deviations for all linear variables by gender and accompanying p values for independent sample t-test of difference by gender.

	Female Mean(SD)	**Male Mean(SD)**	**T-test *p* value**
Skin yellowness (b*)	15.90 (1.54)	15.96 (1.47)	.869
Skin lightness (L*)	67.08 (2.06)	65.20 (1.86)	<.001
Fruit and vegetable portions/day	4.35 (2.56)	4.26 (2.73)	.884
Stress score	4.93 (3.40)	3.74 (2.58)	.139
Godin exercise score	64.41 (25.45)	59.13 (32.25)	.445
% body fat	23.75 (7.77)	12.81 (5.79)	<.001
Alcohol intake (units/ week)	11.34 (12.59)	16.60 (15.16)	.119

Table 2: Frequency counts for nominal factors included in the model

		Gender		
		Male	Female	Total
Smoker status	Smoker	2	10	12
	Non-smoker	21	44	65
	Total	23	54	77

Table 3: Zero order correlations between all linear variables.

	b*	L*	FV/day	Stress	Exercise	Alcohol
b*						
L*	-.407**					
FV/day	.347**	-.045				
Stress	-.253*	.045	.117			
Exercise	.166	.079	.301*	.070		
Alcohol	-.157	-.157	-.214	-.052	.019	
% fat	.108	.180	-.035	.042	-.097	-.025

** $p<.001$; * $p<.05$

The backwards linear regression went through five iterations. The final model significantly predicted skin yellowness F (4, 76) = 11.36, $p < .001$ and was able to account for 38.7% of variance. Both control variables, skin lightness and gender, were retained. Skin lightness showed a negative correlation to skin yellowness and males were found to be less yellow than females (whilst controlling for lightness). Fruit and vegetable intake was retained as a positive predictor of skin yellowness and stress was retained as a negative predictor.

Full results of the first and final model can be viewed in Table 4. Residuals of the final model were normally distributed $D(77)= .04$, $p>.200$. Partial plots from the final model showing the relationship between significant predictors (fruit and veg intake and stress) and skin yellowness can be viewed in Figures 7 and 8 respectively.

Table 4: Predictors of skin yellowness (b*) in cross sectional investigation

	B	**SE B**	**Beta**	***p***
Model 1				
L*	-.35	.07	-.50	<.001
Gender	-.95	.41	-.29	.025
FV/day	.17	.06	.29	.006
Stress	-.15	.05	-.31	.002
Smoker status	-.22	.46	-.05	.635
Exercise	.01	.01	.10	.304
Alcohol Intake	-.01	.01	-.10	.351
Fat	-.02	.02	-.14	.237
Model 5				
L*	-.32	.07	-.46	<.001
Gender	-.70	.34	-.21	.042
FV/day	.21	.05	.36	<.001
Stress	-.15	.04	-.31	.002

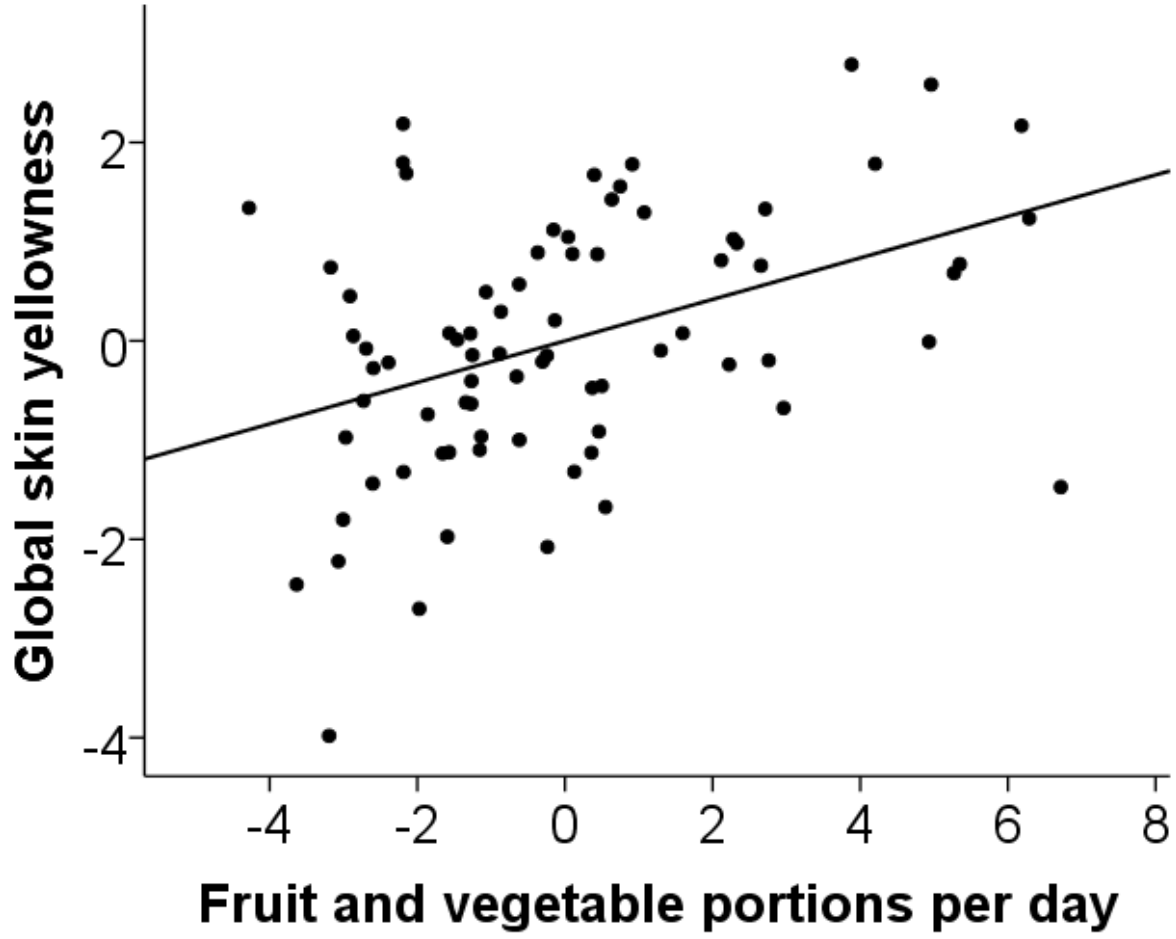

Figure 7: Partial plot showing and positive association between fruit and vegetable intake and skin yellowness whilst controlling for gender, skin lightness and stress. Zero points on each axis represent sample average.

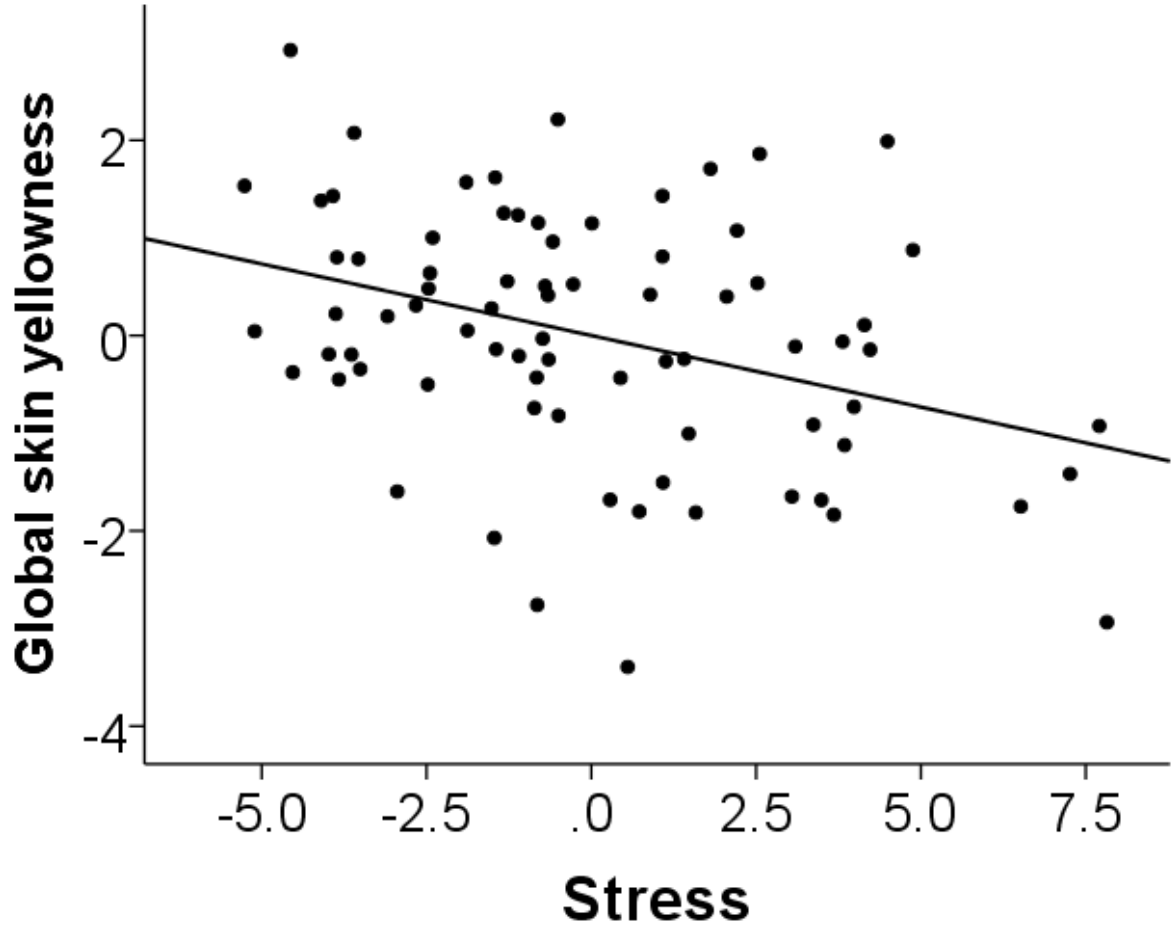

Figure 8: Partial plot showing relationship between stress and skin yellowness whilst controlling for gender, skin lightness and fruit and vegetable intake. Zero points on each axis represent sample average.

3.3.3 Discussion

Results from the regression model confirmed that at least one health risk factor beyond fruit and vegetable consumption is related to carotenoid colouration of human skin. Skin yellowness (b*) was found to be negatively related to psychological stress. The positive relationship between fruit and vegetable consumption and skin yellowness was also confirmed. Both effects were present whilst controlling for skin lightness and so are unlikely to be explained as differences in skin melanin content. No effect of smoker status, alcohol consumption, exercise or percentage body fat was revealed by the analysis.

The finding that psychological stress is related to carotenoid colouration of the skin is in line with literature that psychological stress is related to oxidative stress and suppression of the

immune system, either of which could meditate a relationship between stress and carotenoid colouration of skin. Sivonová and collegaues warned that their noted effect of exam stress upon oxidative stress could have been meditated through behavior change in terms of diet, exercise, smoking or alcohol intake (Sivonová et al., 2004). We found no evidence of zero order correlations between stress and exercise, fruit and vegetable consumption or alcohol intake in the present sample and therefore no evidence that the reported effect of stress was mediated by these behaviours. Rather, stress was found to be a significant independent factor in predicting skin yellowness.

The null results for alcohol, smoker status and body fat, are contrary to prediction; and could have been due to a lack of statistical power. Only a small subsample of participants reported smoking (n=12) and the majority of participants (64%) reported alcohol consumption below the UK recommended guidelines (<14 units per week) with 20% reporting no alcohol consumption at all in the week prior to testing. It is also possible that any effect of alcohol consumption upon skin colour reflect habitual drinking habits and that alcohol consumed in the prior week did not reflect this. With regard to the null effect of fat, we also had a sample of participants who were leaner than the population at large. A minority (18%) of our sample would be considered overweight, with reference to their BMI and WHO guidelines (BMI≥25 considered overweight) while the majority (64.6%) of the Scottish population from which they were drawn are reported to be overweight (Castle, 2015). In support of this interpretation, Bovier and colleagues found a much weaker correlation between BMI and retinal carotenoids in their sample of young healthy adults within the normal range of adiposity levels, relative to that seen in a heavier (approaching obese) population measured by Hammond and colleagues (Bovier et al., 2013; Hammond et al., 2002). There may be other reasons for the null result, for example, in the case of alcohol consumption, it has been suggested that moderate amounts may have protective effects against oxidative stress (Whitfield et al., 2013) and at least some forms of alcohol contain antioxidants (Møller et al., 1996). Another possibility is that a decrease in yellow skin colour due to oxidative stress is masked by an increase in yellowness due to other factors, such as nicotine staining associated with smoking, or bilirubin production associated with alcohol consumption (Whitfield et al., 2013). However, one a large scale epidemiological investigation of lifestyle factors and oxidative stress detailed earlier, found that contrary to their own predictions, there was no measured effects of smoking or alcohol in a sample of over 1700 adults (Trevisan et al.,

2001). Oxidative stress is thought to be a potential mediator of carotenoid colouration, so this study would provide support that our negative results may reflect a true null effect.

Frequency of exercise, also contrary to predictions, did not show any relationship to skin yellowness. This could be because exercise showed a correlation with fruit and vegetable intake, and this correlation was of a similar magnitude to that of fruit and vegetable intake and skin yellowness. In our sample, individuals who exercised frequently also had a higher intake of fruit and vegetables. It may be that there was not enough independent variance in these variables to reliably test independent effects upon skin yellowness.

In addition to fruit and vegetable consumption, only one of the five risk factors investigated showed a significant relationship to skin yellowness, providing limited support for the idea that carotenoid colouration of skin reflects a global measure of condition but demonstrating nevertheless that carotenoid colouration does reflect more than carotenoid intake.

In Study 4, a within subject design is employed to test whether changes in these same health risk factors (excluding smoker status) are associated with a change in skin colour.

3.4. Study 4 – Within subjects investigation

3.4.1. Methods

3.4.1.1. Design

A repeated measures observational design was employed to test how skin colour changed with changes in health relevant variables (stress, fruit and vegetable consumption, alcohol intake, percentage body fat and exercise).

3.4.1.2. Participants

Fifty individuals (23 female, 25 male, mean age 21, age range 18 – 34) participated on two occasions between September and November 2014. Forty-two participants reported being of "White" ethnicity, five reported being of "Asian" ethnicity, two reported "mixed" ethnicity and one participant reported "other" ethnicity.

3.4.1.3. Measured variables

Skin colour

Skin colour was measured as in Study 3, at 6 locations (see section 3.2.1.3). Global measures (an average of all locations) were calculated for each individual at each time point in terms of lightness, redness and yellowness.

Health relevant variables

Stress, fruit and vegetable consumption, alcohol intake and percentage body fat were all measured using the same methods as in Study 3. Exercise frequency was assessed using the International Physical Activity Questionnaire (IPAG) which is similar to the Godin scale (used in Study 3), in that it enquires about vigorous, moderate and mild activities (walking). The IPAQ was favoured because it asks for more details about time spent exercising, asks participants to consider time working in addition to leisure time and provides more modern examples of activities (the full questionnaire can be viewed in Appendix 6).

3.4.1.4. Procedure

Individuals participated on two occasions, six weeks apart. All variables were measured on both occasions.

3.4.1.5. Statistical analysis

Change variables were created for stress, fruit and vegetable consumption, alcohol intake, percentage body fat, exercise, skin lightness and skin yellowness by subtracting scores at time two from those at time one. A backwards linear regression, as in Study 3, was conducted with the dependent variable change in skin yellowness. Predictor variables were all other calculated change variables (stress, fruit and vegetable consumption, alcohol intake, percentage body fat and skin lightness) together with control variables gender and average skin lightness. Average skin lightness was created for each participant based on both time points and was included because we may except to see less change in yellowness in individuals who have a darker skin tone.

3.4.2. Results

Table 5 shows means and standard deviations for all change variables. Zero order correlations between all linear variables can be viewed in Table 6. Zero order correlations between variables did not differ materially when gender was controlled for (see Appendix 5).

Table 5: Descriptive values for change in all predictor variables

	Mean	Standard Deviation	Minimum	Maximum
Skin yellowness (b*)	-.35	.61	-2.13	.614
Skin lightness (L*)	.97	.61	-.90	1.00
Fruit and vegetable portions/day	-.14	1.96	-5.07	1.96
Stress score	1.36	3.45	-10.00	3.45
Physical activity score (MET)	140.10	2110.36	-3723.00	2110.36
% body fat	.09	1.23	-1.80	1.23
Alcohol intake (units/ week)	-.93	14.06	-55.50	14.06

Table 6: Zero order correlations between all predictor variables

	b*	L*	FV/day	Stress	Exercise	Alcohol
L*	-.197					
FV/day	.200	-.116				
Stress	-.295*	-.043	-.065			
Exercise	-.131	.265	-.026	.176		
Alcohol	.119	.273	-.073	.022	-.033	
% fat	.037	-.045	.107	.180	-.173	0.58

**p<.001; * p<.05

The backwards linear regression went through six iterations, the final model significantly predicted change in yellowness F (3, 46) = 9.69, p < .001 and was able to account for 38.7% of variance. Control variables, average skin lightness and gender, were retained. Skin lightness showed a positive correlation to change in skin yellowness i.e., change in yellowness was more prominent in lightly pigmented individuals (β= .43, p = .001). Males were found to show more change than females, whilst controlling for lightness (β= .46, p < .001). Stress was retained as a significant negative predictor (β= -.29, p = .015) but all other predictor variables (including fruit and vegetable consumption) fell out of the model.

Full results of the first and final model can be viewed in Table 7. Residuals of the final model were normally distributed D(50)= .09, p>.200. A plot showing the relationship between

change in stress and change in skin yellowness (whilst controlling for gender and average skin lightness) can be seen in Figure 9.

Table 7: Results of backwards linear regression predicting change in skin yellowness within individuals.

	B	**SE B**	**Beta**	***p***
Model 1				
L* change	-.05	.09	-.08	.588
L* average	.08	.03	.37	.009
Gender	.56	.16	.46	.001
FV/day	.04	.04	.12	.326
Stress	-.05	.02	-.30	.020
Exercise	<.01	.00	.05	.716
Alcohol Intake	.01	.01	.17	.198
Fat	.01	.06	.02	.892
Model 6				
L* average	.10	.03	.43	.001
Gender	.56	.15	.46	<.001
Stress	-.05	.02	-.29	.015

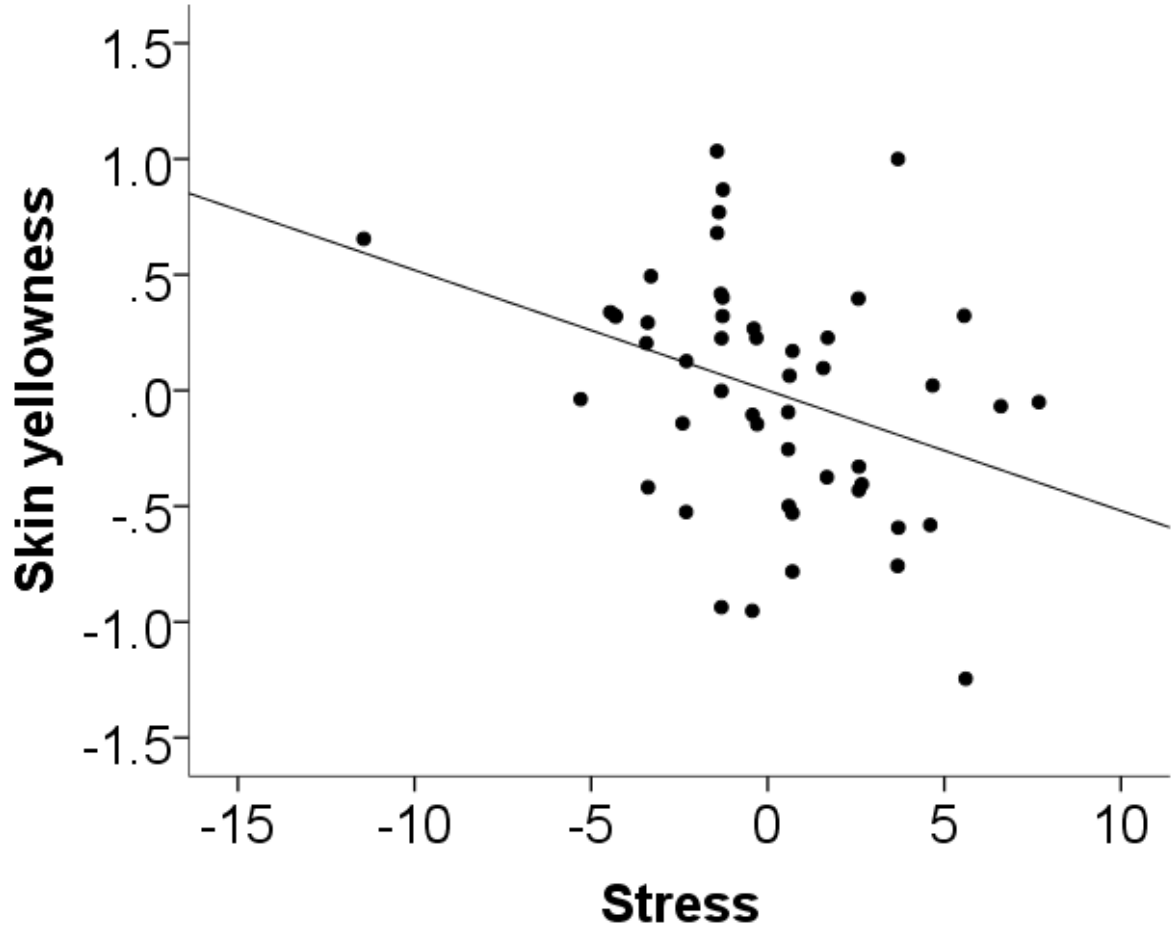

Figure 9: Partial regression plot showing that a drop in skin yellowness co-occurs with an increase in stress whilst controlling for average skin lightness and gender. The correlation remains significant when the individual reporting the largest decrease in stress (>-10) is removed.

Change in lightness was not retained as a predictor for change in yellowness suggesting that the majority of change in yellowness during this time was not driven by changing melanin levels.

3.4.3 Summary

Study 4 showed the same pattern of results to those found in Study 3.Increases in psychological stress were associated with a decrease in skin yellowness and the strength of the relationship was similar in both studies. This relationship was independent of changing melanin levels and of changes in other health relevant variables (fruit and vegetable intake, alcohol consumption, exercise and body fat). Unlike Study 3, Study 4 did not find that a

change in fruit and vegetable intake was related to change in skin colour. This is inconsistent with prior evidence demonstrating such a relationship (Whitehead, Re, et al., 2012), see also Chapter 2. The lack of effect may be due to insufficient variation in changing fruit and vegetable intake. Although some participants reported change in as much as 5 portions per day, the mean value for change was close to zero (-.14) and 68% of cases reported change values of less than two portions per day

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3.5 General Discussion

Both Studies 3 and 4 showed a significant association between skin yellowness and psychological stress. In both studies the association was independent of any differences in skin lightness indicating that the relationship is specific to carotenoids and not melanin. Furthermore, in both studies the effect of stress was independent of other health relevant variables such as exercise, fruit and vegetable consumption, alcohol intake and body fat. The link between stress and carotenoid colouration of skin can therefore not be explained behaviourally by change in any of these other variables. There are however, other behavioural variables that were not tested and could potentially mediate the relationship between stress and carotenoid colouration of skin. These could include sleep, or other aspects of diet (e.g. total caloric consumption). Future work could investigate these additional health relevant variables.

The finding that carotenoid colouration of skin is linked to psychological stress is the first demonstration that carotenoid colouration of human skin reflects an aspect of health beyond carotenoid intake and is important in this sense. However, of the many risk factors to health which were measured, stress was the only one found to show a significant relation to carotenoid colouration. There is therefore limited support for the idea that carotenoid colouration reflects an overall state of condition.

Another possibility however, is that the health relevant variables measured are not the best indicators of current condition in our sample. Risk factors tell us only that an individual is susceptible to disease (whether chronic or acute), but not whether they are currently ill (Rose, 2001). Obesity, lack of exercise, smoking and excessive alcohol consumption may all be unhealthy behaviours that increase one's risk of disease in the future but would not necessarily lead an individual to report being or feeling unwell, or even to report being less healthy, at the time of the experiment. This point may be particularly true in our young

sample of adults were the consequences of such actions are yet to catch up with them. Psychological stress on the other hand, is more likely to be associated with illness at or around the time of the experiment. Physiologically, a stress response will activate both the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) (Herbert & Cohen, 1993), leading to elevated cortisol and adrenaline levels, as well as increased blood pressure and heart rate (Negrão, Deuster, Gold, Singh, & Chrousos, 2000). Stress is often associated with a feeling of being "run down" and as noted earlier has been demonstrated to supress immune system function (Herbert & Cohen, 1993). The reason that stress was the only health risk found to be related to carotenoid colour may be explained if carotenoid colouration of skin is reflecting current condition but many of the measured risk factors have distal rather than proximate effect on condition. Such an explanation is not to suggest that in a more diverse, older or less healthy population, the measured health risk factors would not be relevant to condition and carotenoid colour, and this should be a focus of future investigations.

Regardless of the explanation or potential mediation, that psychological stress is related to carotenoid colouration of skin is an important finding because it confirms that carotenoid content of skin is not solely a function of carotenoid intake. The relationship between stress and carotenoid colour was found both between and within subjects demonstrating its replicability and robustness. Carotenoid colouration of skin is known to be both healthy and attractive looking (Whitehead, Re, et al., 2012), and a demonstration of carotenoid enhanced skin tone has been shown to be an effective motivation to increase fruit and vegetable consumption (Whitehead et al., 2013). The present study therefore has important implications in terms of motivating healthy behaviours and a holistic approach to health because it suggests that stress management may be necessary to protect benefits in terms of health and appearance gained from a healthy carotenoid rich diet.

In summary, psychological stress has been linked to carotenoid colouration, studies found that individuals reporting higher levels of stress had less carotenoid colouration of skin and that when individuals reported an increase in stress, carotenoid colour of skin concurrently decreased. These effects were independent of fruit and vegetable consumption and unrelated to variation in lightness. Other health relevant variables showed no relation to skin yellowness. This could be because there was insufficient variation in these variables to detect effects, or because these were not good indicators of current condition in our young sample; although each of these explanations are not mutually exclusive. Of the measured health risk

variables, psychological stress is most likely to have a proximate effect on the health status of our participants. The suggestion that carotenoid colouration reflects recent or current health of individuals is further explored in the following chapters. Chapter 4 investigates the relationship between skin colour and signs and symptoms of ill health over the prior two months whilst Chapter 5 investigates the effect of experimentally induced illness upon skin colour within a period of 8 hours.

Chapter 4: Self-reported illness

4.1. Chapter outline

Carotenoid colouration in a range of bird species is known to reflect current health. This is evident through studies showing negative correlations between parasite load and colouration or demonstrating a decrease in colour with infection. In humans, carotenoid colouration is visible as a result of carotenoid intake but the relationship between skin yellowness resulting from carotenoids and illness is untested. This chapter presents a two part study investigating the relationship between skin yellowness and self-reported health history. Part one consists of a cross sectional investigation of 83 Caucasian participants and finds that individuals reporting higher frequency and severity of symptoms relevant to infectious disease (e.g. colds and flu) had less yellow skin at the forearm but not palm. Part two, presents results of a follow-up when a subset of participants returned to have variables re-measured two months after initial participation. This within subject follow up finds that when individuals report a two month history of fewer and less severe symptoms of ill health, skin colour is more yellow but yellowness did not differ with current status of health (i.e. in the prior week). Results provide evidence that skin colouration can reflect recent instances of ill-health and probably do so on a timescale relevant to which carotenoids arrive in the uppermost layers of skin

4.2. Introduction

Carotenoid ornamentation in many bird species signals health as evident by a decrease in colouration following exposure to infection. For example male blackbirds show a reduction in carotenoid colouration of the beak following a humoral immune challenge (injection of sheep red blood cells) (Faivre et al., 2003). Red Jungle fowl infected with intestinal nematodes as chicks also showed paler plumage at sexual maturity relative to uninfected peers (Zuk et al., 1990), and treatment of nematode worms will increase comb redness in jungle fowl (Martínez-Padilla et al., 2007); (comb redness has been shown to primarily be the result of dietary carotenoids zeaxanthin and lutein (Perez-Rodruguez, Garcia de Blas, Martinez-Padilla, Mougeot & Mateo, 2016)). House finches too, when experimentally infected with a natural parasite show a reduction in feather brightness and colour saturation, from three weeks post infection (Brawner et al., 2000; Hill et al., 2004). Although two studies testing for colour change with an experimental immune challenge found no change in eye ring colour of red-legged partridges (Perez-Rodriguez et al., 2008) or on colour of skin in domestic chickens (Koutsos, Christopher Calvert, & Klasing, 2003). These studies tested colour change just 48 hours or 24hours (respectively) after an immune challenge which is much sooner than the majority of studies finding significant effects of infection upon colour change.

Observational studies have also found negative correlations between current parasite load and plumage colour. In House finches, the intensity of avian pox infection has been shown to negatively correlate with plumage redness (Thompson et al., 1997). Red grouse with brighter combs also have fewer parasites (Martínez-Padilla et al., 2007)

Carotenoids ornaments also appear to provide an indication of condition or resistance to illness; for example, House finches with redder feathers were found to be more likely to survive a natural epidemic (Nolan et al., 1998) and brighter Zebra finches (resulting from a high carotenoid diet) were able to mount a stronger innate immune response relative to paler individuals when provoked (Blount et al., 2003), as were male greenfinches with brighter feathers (Aguilera & Amat, 2007) and male mallard ducks with brighter bills (Peters et al., 2004).

In a range of bird species then, carotenoid colouration of feathers or beaks is related to recent infection or ability to mount an immune response when challenged. Here, in the present study we address whether human skin colour also reliably relates to prior symptoms of ill health.

The study focuses particularly on those symptoms related to infectious illness (e.g. colds and flu) because all studies referenced above which found links between carotenoids and health in other species did so with reference to acute infections. We hypothesise that in a group of Caucasian individuals, those reporting more frequent and severe symptoms of ill health in the recent past will have less yellow skin, reflecting fewer skin carotenoids.

4.3. Study 5a - Retrospective symptoms: a between subjects investigation

4.3.1. Methods

4.3.1.1. Design

A between subjects observational design was employed to test the relationship between skin colour (dependent variable) and symptoms of ill health during the prior eight weeks (independent variable).

4.3.1.2. Participants and Procedures

Eighty-three Caucasian participants aged 17-26 years (61 females, 21 males, 1 individual failing to report gender) were recruited opportunistically at an outdoor stall during Fresher's Week at the University of St Andrews (between the 8th and 11th of September 2014). All participants had their skin colour measured in two locations (palm and inner forearm) and completed a questionnaire including demographic information, and an abbreviated Symptoms of Illness Checklist (SIC). The location for skin measurements were chosen for convenience. Participants were recruited opportunistically, the palm and inner forearm provided two skin locations which were easily accessible, had low levels of melanin, and would not be compromised by the presence of makeup. The study was approved by the University of St Andrews Human Research Ethics Committee and participants provided prior informed consent.

4.3.1.3. Materials

Skin Colour

Skin colour was measured using a Konica Minolta CM-700d spectrophotometer. Skin colour values were recorded in terms of lightness (L*), yellowness (b*) and redness (a*).

Retrospective symptoms of illness

An abbreviated version of the Symptoms of Illness Checklist developed by Stowell and colleagues was employed (Stowell, Hedges, Ghambaryan, Key, & Bloch, 2009). This questionnaire was selected because it contained a range of symptoms thought to be most relevant to a young and healthy population and has been validated against physician ratings (Stowell, Hedges, Ghambaryan, Key, & Bloch, 2009). Eleven of the thirty-three original items relevant to infectious illness (e.g. colds and flu) were selected for use in the present study (see Appendix 7). The sub-set of questions from the SIC questionnaire were selected for brevity and because prior work showing links between carotenoid colour and illness have done so with reference to transient infectious illness. Participants reported the number of days which each of the 11 symptoms were present during the previous two months in categories from "0 days" to "50-60 days". Participants also reported the impact that each symptom (if present) had on their daily activities, in one of four categories from "did not interfere" to "severely interfered". For each item, frequency score was multiplied by impact score, and a sum of these values provided the final score for retrospective symptoms of illness.

Current Illness

Participants were asked "Do you currently feel ill or have you felt ill in the last week?" And were given three response options "No", "Yes – Currently ill", "Yes – ill in past week".

4.3.1.4. Statistical Analysis

Visual inspection of all variables revealed that scores for retrospective symptoms of illness (but no other variables) were positively skewed, a square root transformation was applied to achieve normality.

For each colour channel (yellowness, lightness and redness), a repeated measures ANOVA was conducted to test whether skin colour (dependent variable) varied as a function of retrospective symptoms (between subject independent variable). Body locations (palm and inner forearm) were included as within subject predictor variables and gender was included as a between subjects control variable (because skin colour is known to differ by gender (Jablonski & Chaplin, 2000) and willingness to report being ill may also differ by gender (Barsky, Peekna, & Borus, 2001)). Where Mauchly's test of sphericity revealed a violation of this assumption, a Greenhouse-Geisser correction was applied.

4.3.2. Results

Scores for retrospective symptoms ranged from 0 to 120 on a scale of 220 points. The average score was 16.82 with a standard deviation of 19.23.

4.3.2.1. Yellowness

The repeated measured ANOVA predicting variation in skin yellowness found a marginal main effect of retrospective symptoms $F(1,79)=3.97$, $p=.050$, $\eta^2_p=.048$ which was qualified by an interaction with measured body location $F(1,79)=7.17$, $p=.009$, $\eta^2_p=.083$.

Post-hoc regression analysis revealed that whilst controlling for gender, retrospective symptoms predicted yellowness at the inner forearm ($\beta= -.30$, $p = .006$; $R^2=.11$, $p=.011$), see Figure 10, but not at the palm ($\beta= -.02$, $p = .855$, $R^2=.00$, $p=.941$).

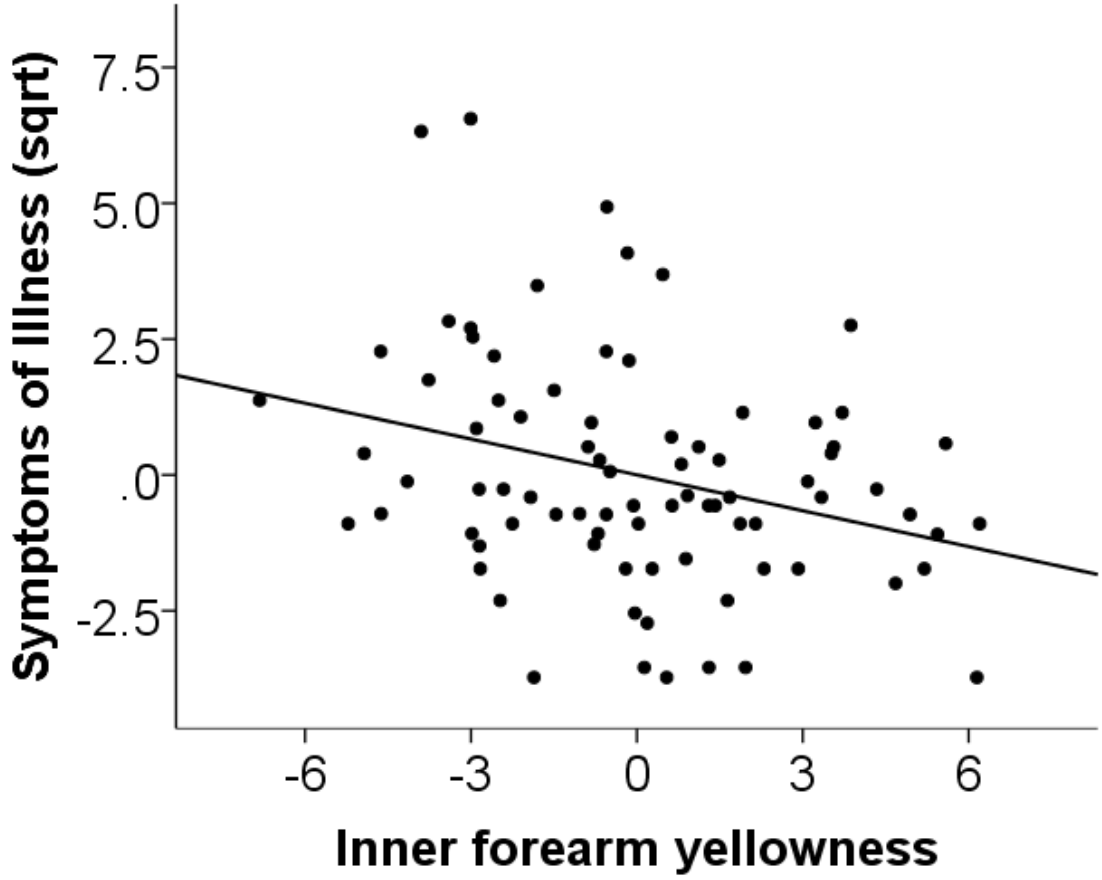

Figure 10: Partial plot showing a negative association between skin yellowness at the forearm and retrospective symptoms of illness whilst controlling for gender. Zero points on each axis represent sample average.

4.3.2.2. Lightness

Retrospective symptoms were not found to predict variation in skin lightness $F(1,79)=1.12$, $p=.293$, the interaction between retrospective symptoms and body location was also non-significant $F(1,79)=2.01$, $p=.160$.

4.3.2.3. Redness

Retrospective symptoms were not found to predict variation in skin redness $F(1,79)=0.01$, $p=.938$, $\eta^2_p=.000$; the interaction between retrospective symptoms and body location was also non-significant $F(1,79)=2.39$, $p=.126$, $\eta^2_p=.029$.

4.4. Study 5b - Change in health: a within subjects investigation

4.4.1. Methods

4.4.1.1. Design

A within subjects, repeated measures observational design was employed to test the effect of change in health (independent variable) upon skin colour.

Change in health was investigated with respect to two separate variables: change in retrospective symptoms (regarding the prior eight weeks); and change in current health (regarding the prior week)

4.4.1.2. Participants and procedures

18 of the 83 participants who took part in Study 5a returned for follow-up eight weeks post original data collection. All measures from Study 5a were repeated.

4.4.1.3. Statistical Analysis

Retrospective symptoms of illness

Sixteen (2 male) of the 18 participants who returned at follow-up scored a different result on the retrospective symptoms of illness questionnaire. Data was reorganised by time of low and high scores for each individual. Those reporting no change in symptoms (i.e. scored the same on both occasions) were omitted from further analysis.

Repeated measures ANOVAs were conducted to test whether skin colour (yellowness, lightness, redness) differed with a change in retrospective symptoms. Within subject

dependent variables were retrospective symptoms (time of high versus low score) and body location (palm and inner forearm).

Willingness to report being ill may differ by gender and could moderate any relationship between change in self-report health and skin colour change. For this reason, it is included as a between subjects variable. However, colour change with illness is not predicted to differ in presence or direction with gender and because the sample of men is small (n=2) gender was included as a control variable but not tested or reported directly.

Current illness

Of the 18 participants who returned at follow-up, 12 (2 male) gave an affirmative answer in response to the question "Do you currently or have you felt ill in the previous week?" on one occasion and negative answer on the other.

Again, data were reorganised by affirmative and negative responses to isolate the within subject dependent variable of change in health status (ill in the past week or not ill in the past week). Those showing no change in current health status (i.e. answered either affirmatively or negatively on both occasions) were excluded from further analysis.

Repeated measures ANOVAs were conducted to test whether skin colour (yellowness, lightness, redness) differed with a change in reported health. Within subject variables were health status (ill versus not ill in past week) and body location (palm and inner forearm). As above, gender was included as a control variable. Where Mauchly's test of sphericity revealed a violation of this assumption, a Greenhouse-Geisser correction was applied.

4.4.2. Results

4.4.2.1. Change in retrospective symptoms (prior 8 weeks)

Change in score for self-reported symptoms of illness ranged from 1 to 74 points (on a 220 point scales) across the two time points. On average, participants reported a change equating to 14.38 points (between the two time points). Eleven individuals reported their highest score for symptom of ill health at baseline and five reported their highest score at follow-up.

Yellowness

The mixed ANOVA showed that whilst controlling gender, there was no significant main effect of retrospective symptoms upon skin yellowness $F(1,14)=3.4$, $p=.085$, $\eta^2_p=.197$ and no interaction with location $F(1,14)=.41$, $p=.530$, $\eta^2_p=.029$. Figure 11 shows that with an

increase in reported symptoms of ill-health, skin yellowness decreases in both locations, but not significantly so.

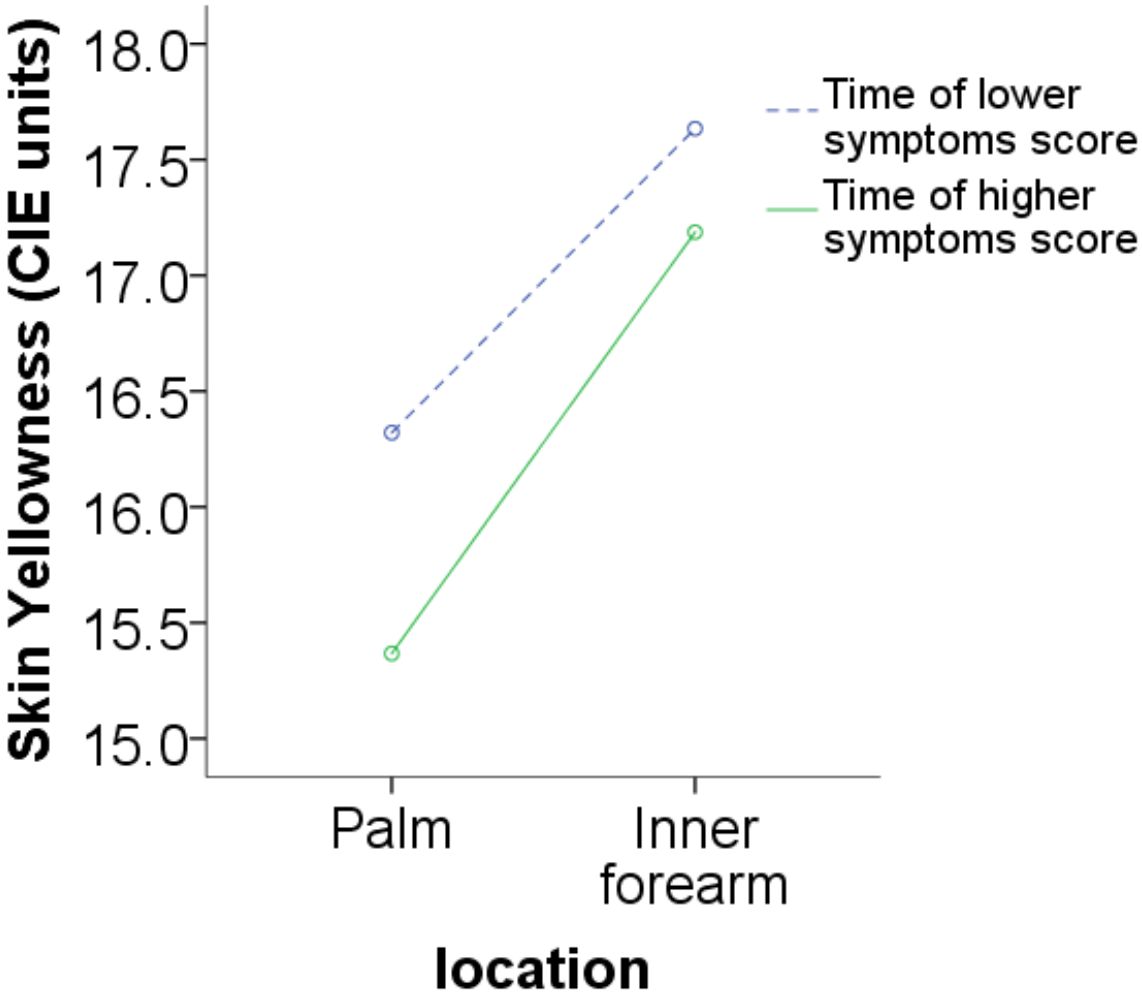

Figure 11: Plot showing estimated marginal means for skin yellowness by location and at times of high and low reported symptoms of ill health whilst controlling gender. Skin yellowness decreases as a function of self-reported symptoms of ill health at both locations (*p*=.085).

Lightness

Skin lightness did not vary with an increase in retrospective symptoms of illness over 8 weeks $F(1,14)=.30$, $p=.592$, $\eta^2_p=.021$ nor was any interaction found between symptoms of illness and measured location $F(1,14)=.20$, $p=.659$, $\eta^2_p=.014$.

Redness

Skin redness did not vary with an increase in retrospective symptoms of illness over 8 weeks $F(1,14)=.26$, $p=.621$, $\eta^2_p=.018$ nor was any interaction found between symptoms of illness and measured location $F(1,14)=.12$, $p=.730$, $\eta^2_p=.009$.

4.4.2.2. Change in Current Illness (prior week)

12 of the 18 participants who returned at follow-up gave a different answer to the question "Do you currently or have you felt ill in the previous week?" and there was an equal split of those reporting ill at each time point.

Yellowness

The ANOVA showed that whilst controlling for gender, there was no significant main effect of current illness upon skin yellowness $F(1,10)=0.29$, $p=.605$, $\eta^2_p=..028$. Nor was there any interaction between current illness and location $F(1,10)=0.00$, $p=.961$, $\eta^2_p=.000$.

Lightness

There was a main effect of current illness on skin lightness $F(1,10)=5.29$, $p=.044$, $\eta^2_p=.346$ but no interaction between current illness and location $F(1,10)=2.33$, $p=.158$, $\eta^2_p=.189$. Skin colour becomes lighter with current illness (see Figure 12).

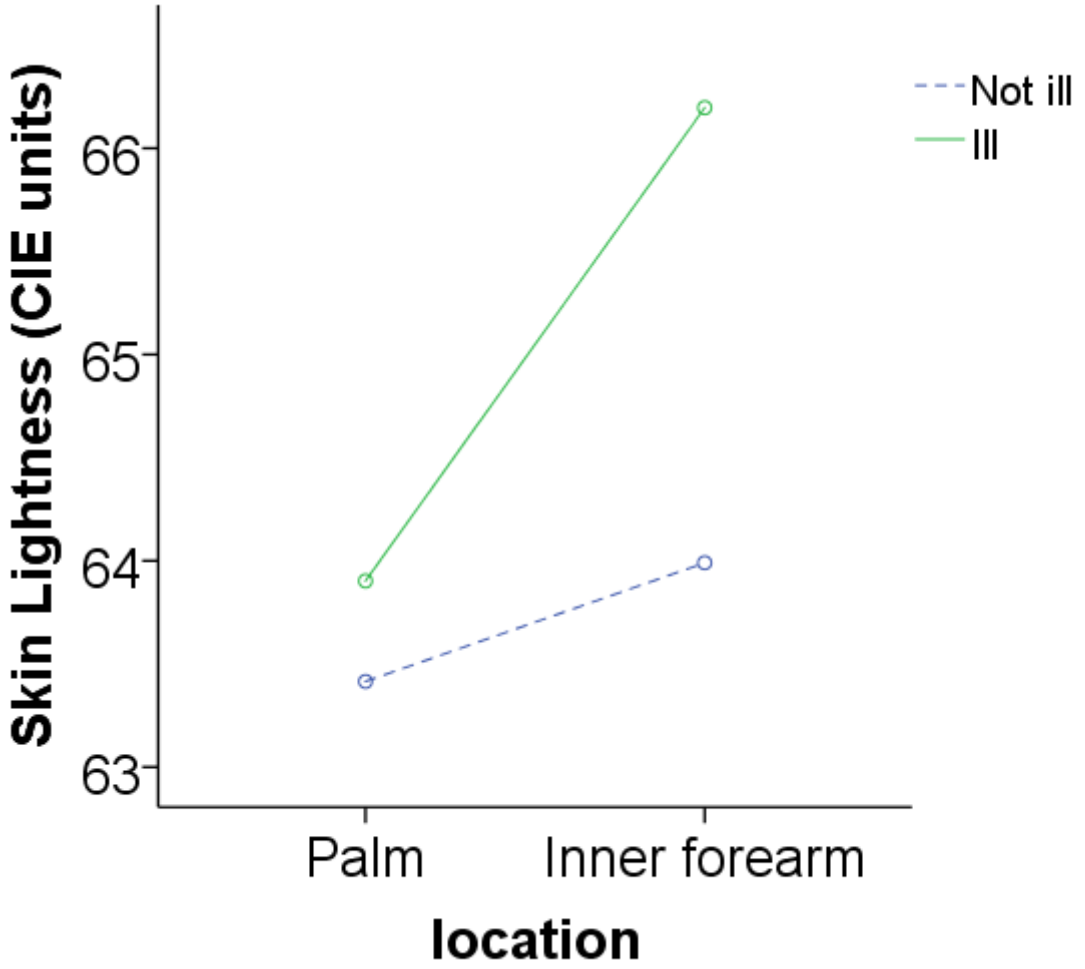

Figure 12: Interaction plot showing that skin lightness increases with current illness (in the past week).

Redness

There was no main effect of current illness on skin redness $F(1,10)=1.97$, $p=.190$, $\eta^2_p=.165$ and no interaction between current illness and location $F(1,10)=.44$, $p=.520$, $\eta^2_p=.043$. Skin redness did not change with current illness.

4.5. Discussion

Results of the current study are the first to demonstrate an association between carotenoid consistent colouration of skin and health history in a group of Caucasian participants. Study 5a found that across participants, skin yellowness measured at the inner forearm was negatively related to the frequency and severity of symptoms associated with infectious illness during the prior eight weeks. In Study 5b it was also found that, within subjects, there

was suggestion of a change in yellowness with a change in health history, skin colour was less yellow following two months in which participants reported a higher frequency and severity of infectious illness symptoms, although this was not significant at the alpha level of 0.95 (p=.085). A change in presence or absence of current illness (in the prior week) was not associated with a change in skin yellowness but did show an increase in skin lightness. The results therefore suggest that illness history over eight weeks is associated with a skin yellowness but there is no evidence that an illness of up to a week affects skin yellowness.

The relationship between skin yellowness and health history both across and between participants is unlikely to be explained as differences in melanin content because there was no concurrent association between reported illness and skin lightness. The link between symptom history and yellowness is therefore best explained by other pigments, most likely carotenoids. Skin lightness did change with current illness (in the last week), where no concurrent change in skin yellowness was found. This effect was not predicted but is consistent with the medical description of "pallor": an unhealthy looking paleness of skin colour. Pallor is usually thought to reflect a reduction in blood flow to the skin (Waring, Monger, Hollingsbee, Martin, & Marriott, 1993). Here we found no evidence for a reduction in redness which would be expected with a drop in blood perfusion. Future work should therefore explore other mechanisms, perhaps skin hydration status.

As noted, no skin colour change consistent with carotenoid loss was recorded with change in current state of health (in the last week). Rather, our results suggest that skin yellowness is related to frequency and severity of illness on a time scale of several weeks. The time frame of carotenoid colour effects is somewhat consistent with studies of carotenoid signalling in birds; bird feathers are often tested for colour change before and after moult (Brawner et al., 2000; Hill et al., 2004), and a drop in beak colour in blackbirds has been reported 3 weeks following an immune challenge (Faivre et al., 2003). Two further studies which have tested for change in carotenoid colourations shortly after an immune challenge (i.e. within 48 hours) (Koutsos et al., 2003; Perez-Rodriguez et al., 2008) found no effect. Carotenoid colouration is presumably reflecting health during the time in which feathers, or keratin cells are replaced. Carotenoid colouration of skin may too be reflecting health status during the period of time in which skin carotenoids are replaced.

The relationship between skin yellowness and health history between subjects, in Study 5a, was limited to measurements at the inner forearm location and no such association was found

at the palm. This is somewhat surprising because carotenoids accumulate in the epidermis (Maeter et al., 2013) and have a greater visual impact in regions where the epidermis is thick, i.e. the palms and soles of the feet. It is likely though, that a reduction in skin carotenoids will also occur more slowly in these regions. Carotenoids can take one of two paths to skin cells; either they defuse from the blood to the lower layers of the skin and make their way to the surface slowly as skin cells are replaced, or they can in effect take a shortcut from the blood to the top layers of the skin through sweat ducts (Darvin et al., 2010). An increase in carotenoids will quickly show in sweaty regions (e.g. the palm) but a drop in carotenoids will take longer to be reflected in the regions of thick epidermis because there are more layers to replace and skin turnover will be slower.

This interpretation of findings is consistent with evidence from Stahl and colleagues who found oral supplementation of carotenoids over 12 weeks was associated with a greater increase in skin carotenoids at the palm relative to the inner forearm; yet 2 weeks after cessation of supplementation, palm skin showed a smaller drop in carotenoids (31%) relative to the inner forearm (47%) (Stahl et al., 1998). In the analysis of change in health history, a marginal change in skin yellowness was seen which was not specific to the inner forearm but present at both locations. The majority of participants reported higher frequency and severity of symptoms at the first time point relative to the second. Therefore, although we investigated a bidirectional change in health history, for the most we captured an improvement. As noted, in the palm carotenoids will accumulate faster than they will be depleted and so this would also explain why we find change in health related to change in palm yellowness with the follow-up but not in the initial cross-sectional investigation. Future work could confirm or refute this explanation by investigating whether reductions in palm colour relates to health history over a longer period of time.

The causal direction of the relationship between symptoms of illness and carotenoid colour was not investigated here and may be explained by one of two interpretations: either that multiple recent bouts of illness led to a depletion of carotenoid resources, or that lower levels of carotenoids left individuals more susceptible to infection. Further research will be necessary to provide support for either of these interpretations.

A limitation of the present study was that carotenoid pigments in the skin were not directly measured but rather inferred because no change in lightness co-occurred. The oxygen saturation of blood will also contribute toward the yellow appearance of skin, with an

increase in deoxygenated blood reducing yellowness (De Felice et al., 2002; Stamatas et al., 2004) and could contribute to the measured relationship between colour and recent illness. However, it is argued that a mediation by carotenoids is more likely than that by deoxygenated haemoglobin for two reasons: firstly, there was no association between illness over the last two months and skin redness or lightness, both of which are more commonly associated with changes in blood content of skin (Stephen, Coetzee, Law Smith, & Perrett, 2009; Waring et al., 1993). Secondly, although decreased blood oxygen saturation is consistent with a number of acute and chronic respiratory infections (Ingram & Munro, 2005; Plüddemann, Thompson, Heneghan, & Price, 2011); in the present study the relationship between skin colour and symptoms of illness was not driven by current state of health, and it is not obvious why current dermal or even systemic blood oxygen saturation should be related to past illnesses.

The demonstrated relationship between skin colour and prior illness has practical implications for the use of spectrophotometry in health screening. Although the current study was retrospective in nature and cannot comment on the causal direction of the relationship between colour and health; research by De Felice and colleagues has shown that b* readings had high predictive accuracy for current illness severity in high risk new-born infants; and were also predictive of survival rates (De Felice et al., 2002). Here we add evidence to the potential value of skin colour readings in healthcare by demonstrating that they are informative of prior health status, and in a relatively healthy sample. Further work may determine whether skin b* values are indicative of an individual's vulnerability to future illness. If so, spectrophotometry measurements could prove valuable in terms of screening individuals for vaccine priority or assessing risk of infection with surgical interventions.

In summary, results of the current study suggest that skin colour at the inner forearm location can be informative of prior health in terms of frequency and severity of symptoms related to infectious disease. Individuals who scored high in terms of symptoms of prior illness were found to have less yellow skin. Findings are consistent with the interpretation that skin carotenoid levels are informative of health history. Replication of this finding will be necessary as will further work determining the causal direction of this relationship, and its generalisability to other measures of health, and other skin types. This is particularly true since the findings may also have practical implications in terms of highlighting the potential value of objective skin colour measurements in healthcare.

Chapter 5: Experimentally induced illness

This chapter is partially based on the following work accepted for publication in the peer-reveiwed journal *brain behavior and immunity*

Henderson, A. J., Lasselin, J., Lekander, M.,Olsson, M. J., Powis, S. J., Axelsson, J., Perrett., D. I. (2016). Skin colour changes during experimentally-induced sickness. *Brain Behaviour and Immunity*. http://dx.doi.org/10.1016/j.bbi.2016.11.008

5.1. Chapter overview

This chapter investigates how skin colour changes with acute onset of sickness and whether any observed changes in colour can be utilised to inform judgements of health. It was predicted that onset of an innate immune response would expend carotenoids leading to a reduction in plasma levels and reduction in skin yellowness. It was also predicted that such a colour change would be perceived as less healthy. In Study 6 skin colour was measured spectrophotometrically in a sample of 22 participants who were injected either with endotoxin to induce an acute immune response, or a placebo saline solution. The study was conducted at medical university with full medical supervision. Colour and plasma carotenoids were measured periodically over an eight hour time frame. Results found that whilst plasma carotenoids were predictive of baseline skin yellowness and showed evidence of a drop in response to acute infection; this drop was not reflected in skin colour change. Skin colour changes were recorded early in response to acute inflammation (1-2 hours post injection). These occurred ahead of a reduction in plasma carotenoids and were characterised by a reduction in redness, and yellowness and an increase in lightness which varied by body location. The largest colour changes were noted in the palm and cheeks and are thought to reflect changes in blood perfusion and oxygenation status. Photographs of participants were also taken at two hours post injection for each condition and averaged to illustrate colour change across the face. Inspection of these images suggested that colour change was marked in the lips.

In Study 7 photographs were presented in a forced choice online experiment whereby raters selected the version that they thought looked healthiest. Faces were presented with eyes and lips blacked out to remove any cues to mood. On separate trials, participants were also presented with isolated lips (standardised for shape) and, patches of skin from the cheek. In all three presentations, raters were able to identify the placebo image as healthier significantly above chance although performance was highest in lip trials. Findings suggest that colour cues associated with acute inflammation are unrelated to carotenoids but can be utilised to accurately inform judgements of health.

5.2. Study 6: Sickness, carotenoids and skin colour

5.2.1 Introduction

Carotenoids are expended with illness. This is known from several investigations of carotenoid signalling in birds (see Chapters 1.5). Chapter 4 also provided some support that illness history is relevant to carotenoid colouration of skin. From the study presented it was not possible to deduce a casual direction of the effect and there was no evidence that current illness was related to carotenoid colouration of skin. However, there is some evidence from studies using Raman spectroscopy methods, that human skin carotenoids can fall within 30 minutes (in response to vigorous exercise: (Vierck et al., 2012)). The effects of psychological stress on carotenoids is also claimed to be detectable within 24 hours (Lademann et al., 2014) (although see Chapter 1.5 for a critical review of these findings). In some studies of birds, plasma carotenoids have been shown to fall rapidly i.e. within 6 hours following an immune stimulation in Eurasian and Lesser kestrels (David Costantini & Dell'Omo, 2006; Rodríguez, Broggi, Alcaide, Negro, & Figuerola, 2014); although changes in colouration are usually delayed, on a scale of days to weeks (Perez-Rodriguez et al., 2008). There is precedent then for carotenoid content of skin to change rapidly in response to illness. No such evidence was seen in Chapter 4, although a limitation of the test for current illness was self-report data and the vague nature of the question "Do you currently feel ill?" which may have been interpreted differently by individuals. Here, in Study 6, we employ an experimental model of acute illness to test whether plasma carotenoids are reduced and whether this is reflected in skin colouration.

A reduction in carotenoid colouration is not the only change we expect to see in skin colour with experimentally induced sickness. Variation in skin tone, particularly in the face has long been recognised as a sign of acute illness. This relationship is characterised by phrases such as looking "off-colour", "pasty" or "peelie-wally": a Scottish adjective used to describe sickly-looking paleness of skin. We therefore also aim to investigate more generally how skin colour varies with experimentally sickness.

An intravenous injection of bacterial endotoxin (i.e. lipopolysaccharide, LPS) is a well-established model of acute human sickness. LPS elicits a temporary innate immune response characterised by a release of pro-inflammatory cytokines (including Tumour Necrosis Factor alpha (TNF-a), and Ineterleukin-6 (IL-6)). At high doses (i.e., 4ng/kg body weight) the cytokines stimulated by LPS induce flu-like symptoms including fever, chills, headaches,

nausea and general feeling of malaise (Benson, Engler, Schedlowski, & Elsenbruch, 2012). We use this model to investigate plasma carotenoids and skin colour changes with acute sickness. It is predicted that with illness plasma carotenoids will fall and that the skin will become less yellow, less red, and lighter. We also explore the time-course and topographical variation in skin colour change to further understand the nature of observed colour changes. Time course of colour change is compared with those of measured physiological and psychological responses to acute inflammation (cytokine response, body temperature and self-reported sickness).

5.2.2. Methods

5.2.2.1. Design

A within-subjects experimental design was employed to test the effects of LPS (relative to placebo) on cytokine response, body temperature, self-reported sickness, skin colouration, and plasma carotenoids. Both participant and experimenters were blinded to condition and order was counterbalanced over two sessions.

5.2.2.2. Participants and Procedures

Twenty-two Caucasian participants (9 female, mean age 23.4 years, SD 3.5 years) were recruited by advertisements on University campuses and high schools in the Stockholm area. The study was conducted at Danderyd Hospital within Karolinska Institutet, a medical university where collaborators worked closely with medical staff and have expertise in using LPS as an experimental model of sickness. The sample size was determined with reference to previous experiments showing strong effects of LPS on inflammatory cytokines at the dosage given in the present study or less (Karshikoff et al., 2015; Sundelin et al., 2015). Exclusion criteria were related to age (those under 18 or over 50), body mass index (less than 18.5 kg/m^2 or greater than 30 kg/m^2), smoking, excessive use of alcohol and anyone with a diagnosed physiological or psychiatric disease. Participants gave informed consent and all procedures were medically supervised and reviewed by the regional ethical review board in Stockholm, Sweden.

Volunteers participated in two sessions receiving an intravenous injection (administered by medical staff) of either LPS (*Escherichia coli* endotoxin, Lot H0K354 CAT number 1235503; 2.0 ng/kg body weight) or saline (NaCl 0.9%, placebo condition) (see also Karshikoff et al., 2015; Sundelin et al., 2015 for similar methods). Throughout the following 7.5 hours, measurements of plasma cytokines (IL-6 and TNF-α), body temperature, self-

reported sickness, skin colour and plasma carotenoids were performed. Photographs were also taken before, during and at the end of the experiment. The timing of these measures is summarised in Table 8.

Table 8: Experimental timeline summarising when measurements of all variables were collected

Measured Variable	**Time Post Injection (hours)**									
	0	0.5	1	1.5	2	3	4	5	7	7.5
Cytokines	✓		✓	✓	✓	✓	✓	✓	✓	
Temperature	✓	✓	✓	✓	✓	✓	✓	✓	✓	
Sickness	✓			✓		✓		✓	✓	
Colour	✓		✓		✓	✓	✓	✓		✓
Carotenoids	✓		✓			✓			✓	
Photographs	✓				✓					✓

5.2.2.3. Measures

Cytokines

Plasma concentrations of IL-6 and TNF-α were assessed using multiplexed luminex assays (Human Mag Luminex Performance Assay, LHSCM000, LHSCM206, LHSCM210, RnD Systems, MN, USA) according to the manufacturer's instructions.

Self-reported Sickness (SQ-score)

A 10 item self-report questionnaire of perceived sickness was administered in Swedish. A translation of the items reads: 1) I want to keep still; 2) my body feels sore; 3) I wish to be

alone; 4) I don't wish to do anything at all; 5) I feel depressed; 6) I feel drained; 7) I feel nauseous; 8) I feel shaky; 9) I feel tired; 10) I have a headache. Items were rated from 0 to 3 in terms of agreement and summed to provide a sickness score (SQ-score). This questionnaire was selected because it is short and has it has been validated against an experimental model of sickness in the language which it was administered in here (Andreasson et al., 2016)

Skin Colour

As in prior experimental chapters, skin colour was measured in terms of lightness (L*), redness (a*) and yellowness (b*) using a Konica Minolta CM-700d, a Spectrophotometer. At each time point (see Table 5), measurements were taken across 6 locations (palm, inner forearm, shoulder, left and right cheek, and then forehead). Each location was measured twice and values for each colour channel (L*, a*, b*) were averaged across the 2 measurement iterations. The two cheeks measures were averaged again to provide one cheek variable. Global L* a* and b* values were also created by averaging across palm, inner forearm, shoulder, cheeks and forehead.

Plasma Carotenoids

Plasma samples were analysed by Craft Technologies (City, State, USA) by high performance liquid chromatography (HPLC). Plasma concentrations of lutein, zeaxanthin, lycopene and beta-carotene were summed to give a value of total carotenoids for each participant at each sampled time point (T0, T1, T3, T7.5) in both conditions.

Photographs

Photographs were taken at baseline, 2 hours post injection of LPS or placebo, and 7 hours post LPS. All participants were sat at an equal distance from the camera and asked to maintain a neutral expression. All makeup and jewellery was removed and clothes were covered to prevent colour reflecting onto the face.

To illustrate the difference in facial appearance following injection of either LPS or placebo saline solution, composite images were created from photographs taken 2 hours post injection. Each composite consisted of the same 22 individuals photographed two hours post injection. In *PsychoMorph*, 184 facial landmarks were placed on each image before composites displaying the average shape, colour and texture were created.

5.2.2.4. Statistical Analysis

Data preparation – missing values and transforms

Data were missing for all time points at the inner forearm and shoulder of three participants who were extensively tattooed, for these individuals global measurements are based on 4 rather than 5 locations. Given the within subject design of the study and analysis this difference in computation of global colour measures will not affect interpretation of results.

Of remaining participants and locations, 18 (0.8%) data points were missing in the LPS condition (all locations from 1 participant at T4 plus shoulder for 1 participant at T7.5). Fifteen (0.7%) data points were missing in the placebo condition (all locations from 1 participant at T4). These missing values were replaced with values extrapolated by averaging colour values from the relevant individual and location for the time point immediately prior and subsequent (for T4); or continued solely from the prior time (for T7.5).

Cytokine concentrations (IL-6 and TNF-α) were missing at one of eight time points for two participants in the LPS condition and at one of eight time points from a different two participants in the placebo condition because blood samples could not be taken at this moment. No imputations were made for missing values because values were missing at critical time points around peaks, where missing values could be increasing or decreasing. Cytokine data were log transformed to reduce negative skew.

Validation – Does skin yellowness reflect plasma carotenoids?

Simple correlations were employed to test whether global skin yellowness, redness or lightness correlated with baseline total carotenoids. For colour channels showing a significant global correlation with plasma carotenoids, location specific correlations were tested for each body location measured. These analyses were conducted upon data collected in the LPS condition and then replicated using data from the placebo condition. Residuals from all correlations were normally distributed (Kolmogorov-Smirnov test confirmed all ps>.05).

Effects of LPS on plasma carotenoids, colour, physiology and self-reported sickness

A series of 2 way ANOVAs were conducted to test whether there was a significant condition (LPS vs placebo) by time (7 time points sampled) effect for all dependent variables, including colour at each measured body location. A Greenhouse-Geisser correction was applied in instances when the assumption of sphericity was violated.

To aid interpretation of interactions, the difference between conditions (LPS relative to Placebo) for each dependent variable was plotted over time. Error bars on all plots represent 95% confidence intervals around the paired difference and allow inferences to be made as to where differences are significant at an alpha level of .05 (Pfister & Janczyk, 2013). Where error bars do not cross the x-axis, the difference between conditions can be considered significantly different from zero, i.e. there is an effect of condition. Additionally, where error bars do not overlap, the difference between conditions at any two time points can be considered significantly different.

5.2.3. Results

5.2.3.1. Confirmation of Manipulation

Cytokines (IL-6 and TNF-α), temperature and self-reported sickness all showed significant interactions between condition and time suggesting that these variables change differently over time in response to LPS (all $ps < .001$, all $\eta p^2 > .633$). Figure 13 shows the change in each of these variables over time and confirms that none of these variables differed by condition at baseline (prior to injection), all increased thereafter to a peak between 2 and 3 hours post injection before then falling towards baseline levels. This pattern confirms expected effects of the manipulation.

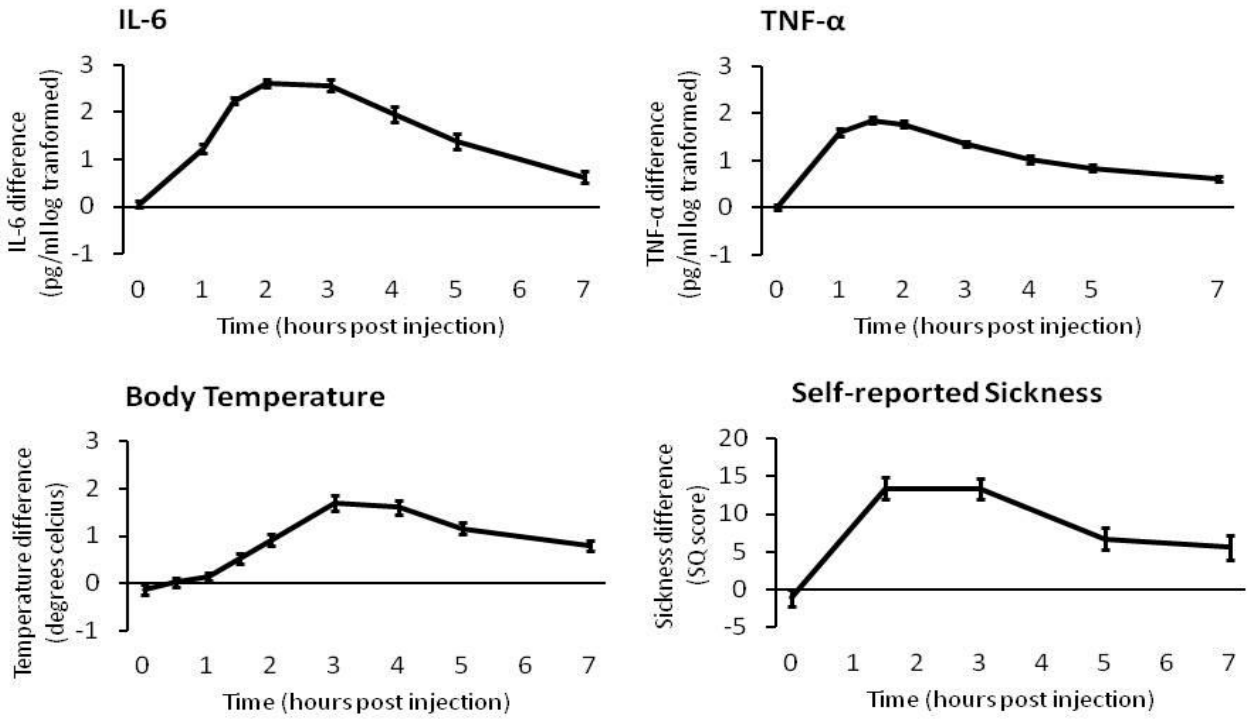

Figure 13: Change in cytokines, body temperature and self-reported sickness over time in response to LPS. Error bars represent 95% confidence intervals around the paired difference (LPS minus placebo)

5.2.3.2. Validation – Does skin yellowness reflect plasma carotenoids?

In the LPS condition, baseline plasma carotenoids correlated with skin yellowness ($r(22)=.61$, $p=.003$) but not skin lightness ($r(22)=.-.11$, $p=.628$) or redness ($r(22)=.24$, $p=.281$). Figure 14 shows the relationship between baseline plasma carotenoids and global skin yellowness.

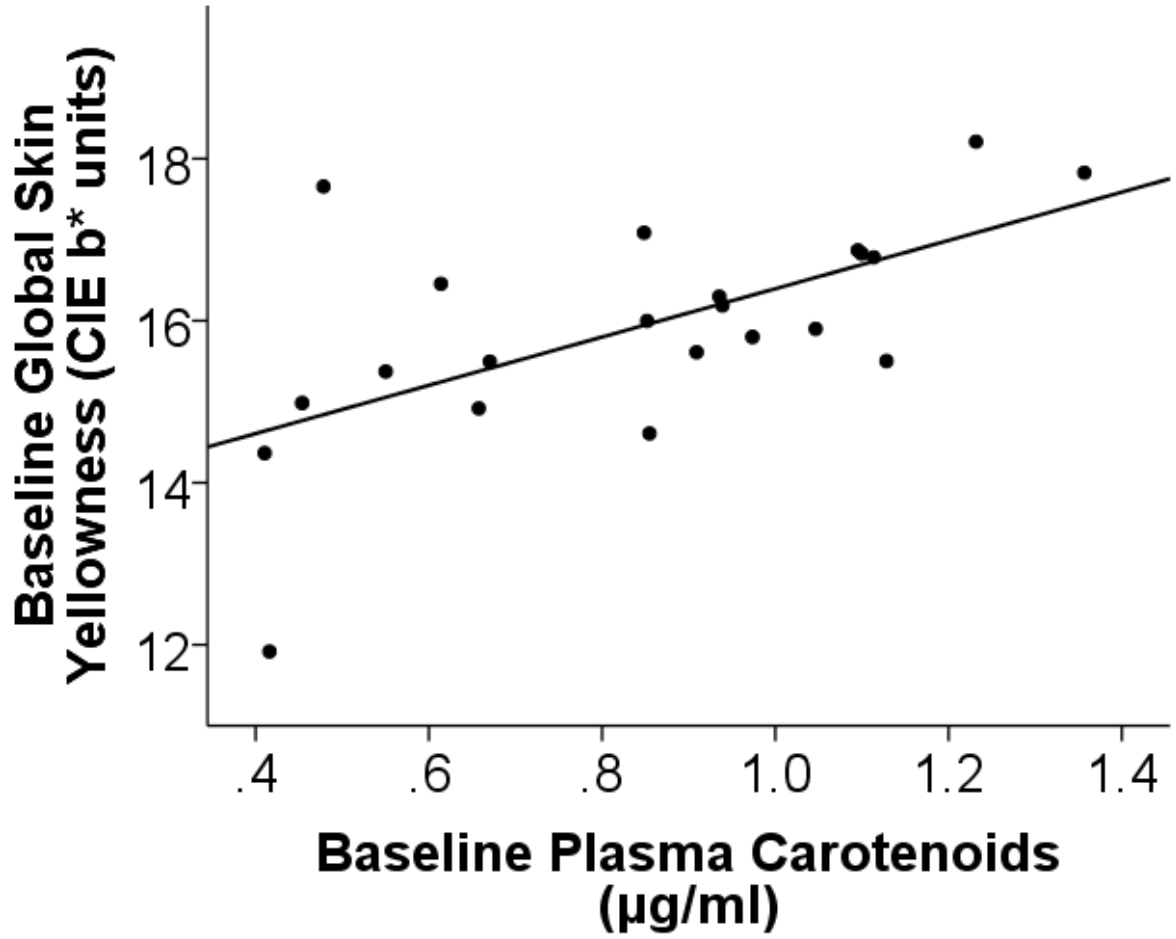

Figure 14: Scatterplot showing a positive relationship between baseline plasma carotenoids and skin yellowness across participants (*n*=21).

A replication of this analysis with data from the placebo condition showed the same pattern of results with baseline plasma carotenoids correlating with skin yellowness ($r(21)=.59$, $p=.005$) but not skin lightness ($r(21)=.19$, $p=.414$) or redness ($r(21)=-.26$, $p=.262$).

A post-hoc 2 step hierarchical regression (for each condition) demonstrated that including skin lightness as a second predictor (to control for melanin) did not significantly improve the strength of correlation between skin yellowness and plasma carotenoids (LPS: R^2 *Change*=.012, $p=.556$; Placebo: R^2 *Change*=.028, $p=.380$)[2]

[2] This was a small homogenous sample of lightly pigmented individuals. Mean Lightness: 66.92, SD: 1.95. In a sample of more darkly pigmented or varied individuals lightness would be a more necessary control.

By location, plasma carotenoids were found to correlate most strongly with yellowness of the palm, closely followed by the shoulder. There was also some evidence that cheek yellowness was related to plasma carotenoid concentration although other locations (inner forearm and forehead showed no relationship to plasma carotenoids). Results of all correlations can be seen in Table 9.

Table 9: Location specific correlations between baseline plasma carotenoids and skin yellowness.

		Palm	Inner forearm	Shoulder	Cheeks	Forehead
LPS	*r*	.579**	.313	.562**	.387	.285
	p	.005	.179	.010	.075	.199
Placebo	*r*	.699**	.196	.675**	.450*	.072
	p	<.001	.421	.002	.041	.756

5.2.3.4. Plasma carotenoids and LPS

A 2-way repeated ANOVA revealed a significant interaction between condition and time $F(3,60)=9.78$, $p<.001$, $\eta p^2 = 328$ in predicting plasma carotenoid levels. Figure 15 shows the difference in plasma carotenoids between conditions at each time point. Error bars suggest that at 7 hours post injection, LPS was associated with a significant drop in plasma carotenoids but that levels did not vary by condition at any other time point.

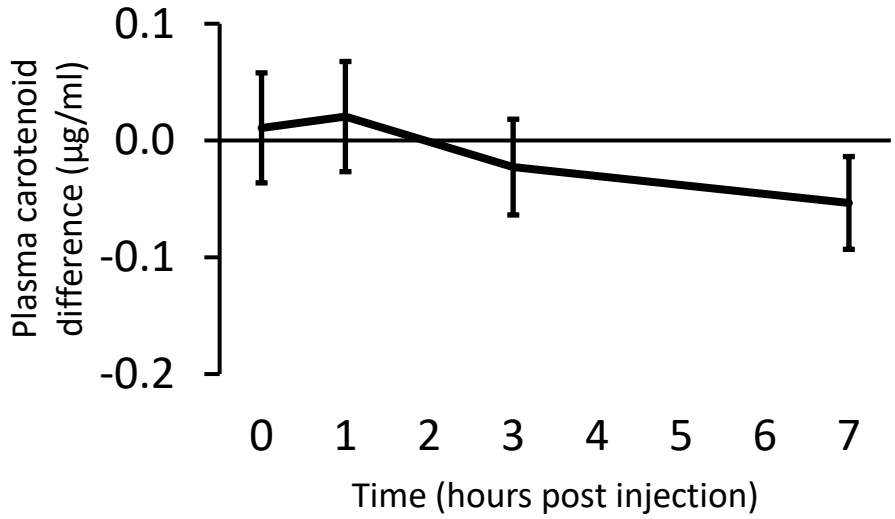

Figure 15: The difference in plasma carotenoids with LPS (relative to placebo) over time. Error bars represent 95% confidence intervals around the paired difference and suggest that carotenoids are significantly lower with LPS 7 hours post injection.

5.2.3.5. Skin Yellowness and LPS

2-way ANOVAs (condition (2 levels) x time (7 levels)) for each location revealed interactions between condition and time at the palm ($F(3.37,70.08)=6.30$, $p<.001$, $\eta p^2=.23$), shoulder ($F(6,114)=3.77$, $p=.002$ $\eta p^2=.17$), cheeks ($F(3.37,70.08)=4.15$, $p=.005$, $\eta p^2=.17$) and forehead ($F(6,126)=2.70$, $p=.017$, $\eta p^2=.11$) but not at the inner forearm ($F(6,114)=1.64$, $p=.143$, $\eta p^2=.08$). At locations showing significant interactions, skin yellowness can be assumed to change differently over time in response to LPS and placebo.

For each location the difference in skin yellowness by condition over time can be viewed in Figure 16 which shows that yellowness did not differ by condition at baseline. Over time, the palm, shoulder and forehead show a clear drop in skin yellowness with LPS 1-2 hours post injection. The inner forearm also appears to shows a slight drop in yellowness with LPS 1-2 hours post injection despite a non-significant interaction term for this location. Paradoxically, the cheeks showed an increase in yellowness 2 hours post injection.

5.2.3.6.. Skin Redness and LPS

2-way ANOVAS (condition x time) for each location revealed interactions between condition and time at the palm ($F(3.97,83.26)=12.29$, $p<.001$, $\eta p^2=.37$), inner forearm ($F(3.01,57.11)=3.99$, $p=.012$, $\eta p^2=.17$), cheeks ($F(3.95,82.89)=8.26$, $p<.001$, $\eta p^2=.28$) and

forehead (F(3.63,76.21)=5.58, p=.001, ηp^2=.21) but not at the shoulder (F(6,114)=0.65, p=.694, ηp^2=.03). At locations showing significant interactions, skin redness can be assumed to change differently over time in response to LPS and placebo.

Figure 16 shows a clear drop in skin redness at the palm, cheeks and forehead within 1 hour of LPS injection. The drop is greatest and longest lasting in the cheeks which do not appear to fully recover to baseline levels until 7 hours post injection. Forehead redness recovers within 3 hours and palm within 4. The inner forearm which showed a significant condition by time interaction was redder in the LPS condition at baseline. Error bars suggest that there is not a clear effect of LPS upon skin redness here.

5.2.3.7. Skin Lightness and LPS

2-way ANOVAS (condition x time) for each location revealed interactions between condition and time at the inner forearm (F(3.99,75.76)=6.59, p<.001, ηp^2=.26) and cheeks (F(6,126)=6.48, p<.001, ηp^2=.24) only. Skin lightness did not change differently over time in response to LPS and placebo at the palm (F(3.73,78.38)=1.96, p=.114, ηp^2=.09), shoulder (F(4.27,91.14)=1.74, p=.146, ηp^2=.08) or forehead (F(6,126)=0.95, p=.460, ηp^2=.04).

Figure 16 shows that the cheeks became lighter 1-3 hours post LPS injection whilst the inner forearm became lighter 1-2 hours post LPS injection.

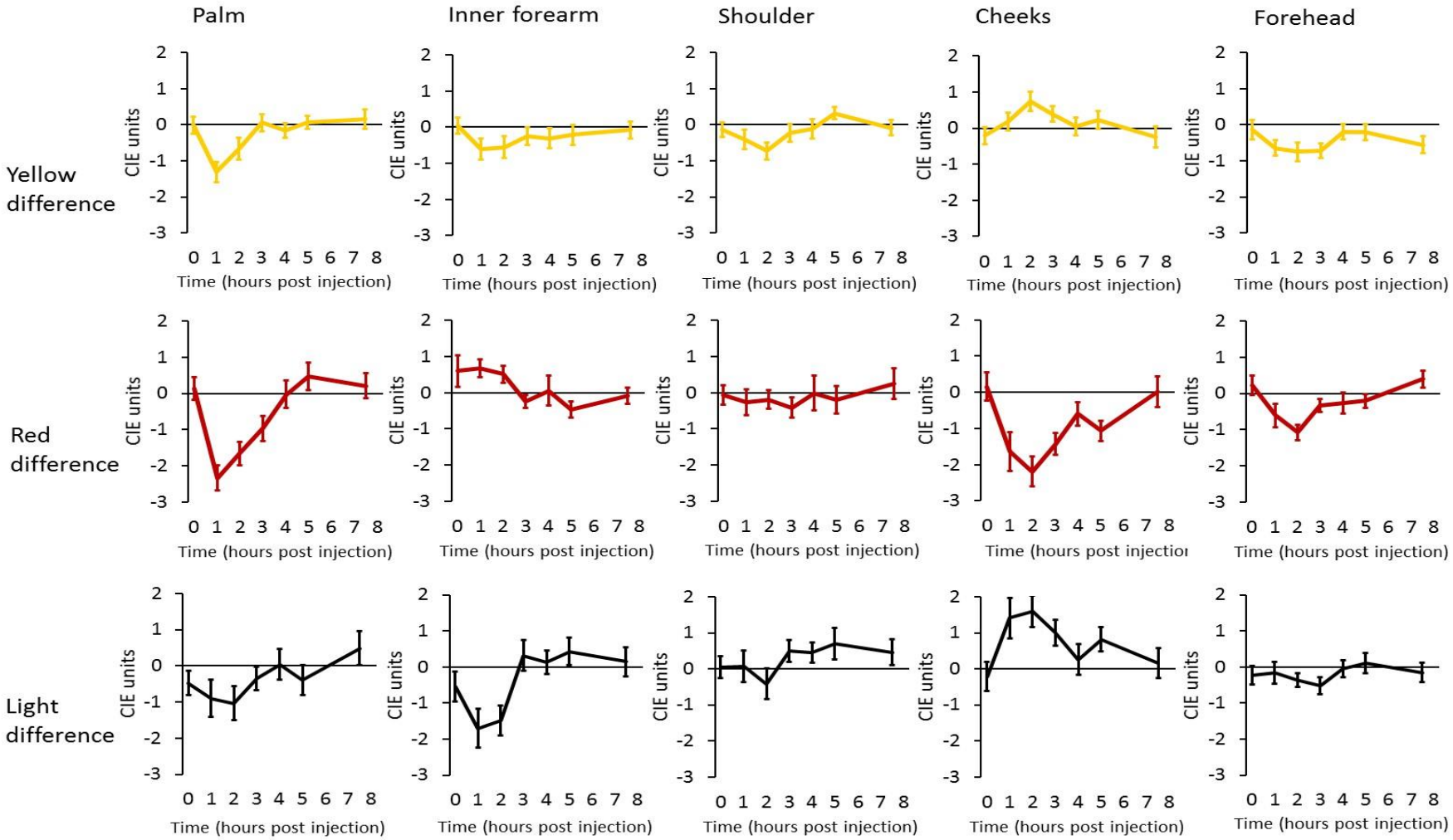

Figure 16: Colour change with LPS over time and by location. Error bars represent 95% confidence intervals around the paired difference (LPS minus placebo). Where error bars do not cross the x axis, colour change is significantly different by condition. Where error bars do not overlap, change with LPS is different by time point.

Figure 17 provides a visual illustration of colour change across the face in response to LPS. Images show the average face of all 22 participants 2 hours post injection of LPS or placebo. Colour change appears to be fairly uniform, somewhat more pronounced in cheeks, and lips show a clear difference.

Figure 17: Averaged images of all participants 2 hours post injection of LPS (left) or placebo (right).

5.2.4. Discussion

5.2.4.1. Carotenoids and illness

Correlations between baseline plasma carotenoid levels and skin yellowness confirm that prior to the any manipulation; skin colouration did reflect carotenoid status of the individual, this was true globally, but driven by significant associations at the palm and shoulder locations. These findings are consistent with prior work showing that plasma carotenoids correlated with skin carotenoids (Stahl et al., 1998) and the skin carotenoid content is reflected in terms of skin yellowness (Alaluf, Heinrich, et al., 2002) (see Chapter 1.3).

In response to LPS, plasma carotenoids showed a significant reduction relative to the placebo condition at seven hours post injection. This finding is consistent with the assumption that carotenoids are expended with illness and specifically with the acute inflammatory response typical of sudden onset infection (Beutler, 2009). The reduction in plasma carotenoids

associated with LPS occurred later in time relative to key changes in cytokine production, body temperature change and self-reported sickness all of which began to recover before any significant drop in plasma carotenoids. This suggests that plasma carotenoid loss may be related to their function as antioxidants, helping the body to recover from the oxidative stress induced by the activation of the immune system.

The noted reduction in plasma carotenoids was not reflected in skin colouration; at 7.5 hours post injection, the difference in skin yellowness by condition had returned to baseline levels at all measured locations. Several locations did show an early drop in skin yellowness around 2 hours post injection, but this recovered before the measured drop in plasma carotenoids. Other researchers have reported a rapid decrease and recovery in skin carotenoid levels using Raman spectroscopy (Vierck et al., 2012). However, in the present study, if the early drop in skin yellowness were assumed to be active recruitment of carotenoids from the skin, it is not clear how these could be replenished without plasma levels also showing a rebound on a similar timescale. Carotenoids arrive in the skin from the blood either by diffusion through lower layers of skin, or through sweat glands (Darvin et al., 2010). It is therefore very unlikely that the drop in skin yellowness measured is indicative of any change in skin carotenoid levels. We must conclude that although the acute inflammatory response is associated with a depletion of carotenoid resources, this depletion is not reflected in skin colouration within eight hours. Given that baseline plasma carotenoids correlate highly with skin yellowness, it is likely that prolonged or more severe illness would be reflected in carotenoid colouration of skin.

5.2.4.2. How else might colour changes with sudden onset illness be explained?

Skin yellowness was not the only colour axis to show early change around 2 hours post LPS injection, a reduction in redness was pronounced in the palm, and face (cheeks and forehead). Changes in lightness were also noted with inner forearm skin becoming darker and cheeks becoming lighter.

Change in redness showed the largest effect (particularly in the palm and cheeks) and peaked around the same time as cytokine production and self-reported sickness but slightly ahead of peak body temperature. Given that maximum body temperature closely followed maximum drop in redness, it is likely that the colour change reflects a reduction in peripheral blood perfusion, as part of the body's attempt to conserve heat during the early stages of fever

production (Anochie, 2013). This change would have been most obvious in lightly pigmented body regions (the palm) and those above the heart (the face) as blood drained away with gravity.

The change in skin yellowness could be explained in relation to changes in oxygenation of the blood. As supply of fresh oxygenated blood to the skin decreased, we can expect a decrease in redness, as recorded, but also a relative increase in the quantity of deoxygenated blood remaining. Deoxygenated haemoglobin reflects more blue light relative to oxygenated haemoglobin and so a shift in the oxygenation status of blood is consistent with a drop in yellowness (given that b* values represent a scale from blue to yellow). Deoxygenated blood is also darker and where skin also dropped in lightness (the palm and inner forearm) this may reflect pooling of oxygenated blood. Blood will also appear bluer in deeper levels of the skin regardless of oxygenation status because shorter wavelengths do not penetrate as deeply into the skin as longer wavelengths (Kienle et al., 1996). If blood is withdrawn from superficial layers, long wavelengths (e.g red light), will still reach it deeper in the tissue, here it will be absorbed, and so not reflected. Meanwhile, less blue light will reach the blood and so will not be absorbed, it will instead be scattered or reflected in higher levels giving the skin a more blue appearance. This may explain loss of yellowness where no drop in lightness occurred (the forehead).

The cheeks, unlike other locations, showed an increase in yellowness and lightening of the skin. The cheeks are special because they contain a high volume of arteries with enhanced ability to dilate and/or constrict (Jablonski, 2013). This would have made blood loss particularly notable here. They also have high levels of subcutaneous fat. The draining of blood from this location could lead to the increase in lightness with blood pigment loss and may have revealed the colouration of the underlying fatty tissue which is yellow.

Visual inspection of average images suggests that lips show a notable colour difference with LPS. Colour difference elsewhere appear to be uniform but slightly more pronounced in the cheeks. In addition to colour, subtle differences in expression can be seen, particularly around the mouth which appears to be more downward turned with LPS.

In summary, skin colour changed in response to LPS 1-2 hours post injection and occurred around the same time as cytokine response but ahead of maximum temperature. Although there was variation in the appearance of colour change across body locations, potentially all the changes can be explained with reference to a reduction of peripheral blood perfusion and

relative increase in deoxygenated blood. The manifestation of these physiological changes in terms of skin colour will depend on a number of factors; particularly the height of the measured location in relation to the heart, and the fat content of the skin. Other factors may also be important such as the melanin content or thickness of the skin. From visual inspection of averaged images, the lips seem to be an obvious region for colour change. Plasma carotenoids were found to fall in response to LPS yet these changes do not appear to be reflected in skin colour change within eight hours.

5.4. Study 7: Perception of illness from skin colour

5.4.1. Introduction

A face flush with colour is attributed with good health and may serve as a cue for mate choice. Conversely, a distinct lack of colour in the face could serve as a cue to ill health also prompting actions from others in terms of disease avoidance or care provision. Perceptual studies have shown that faces with slightly raised levels of red and yellow colour are judged as looking healthier (Stephen, Law Smith, et al., 2009; Stephen et al., 2012). The direction of these colour preferences (higher levels of red and yellow judged as healthy) is consistent with the colour changes measured with experimentally induced illness in Study 6 (when injected with LPS, skin became less red and less yellow). Skin luminance (high lightness values) have also been associated with increased judgements of health (Stephen, Law Smith, et al., 2009), which is inconsistent with our findings showing an increase in lightness at the cheeks with LPS. Yet Stephen et al. (2009) showed only that lightness was preferred when other colour axis were held constant or yellowness was simultaneously increased. Our findings show a drop in redness and yellowness with increased lightness and this combination of colour change has not yet been tested in terms of perceived health or attractiveness. Whether the observed change in skin colour with activation of the immune system as witnessed in Study 6 is sufficient (and utilised) to inform judgments of health needs to be tested empirically, which is the purpose of Study 7.

Study 6 demonstrated that colour change was not evenly or consistently distributed across the face. For example, the forehead showed a reduction in yellowness, whilst the cheeks showed an increase in yellowness and a greater reduction in redness. Further, in average images, the lips stand out as notably different in colour but prior studies of perceived health have

generally applied a uniform colour transformation to skin within whole faces whilst keeping lips colour constant (Lefevre & Perrett, 2014; Spisak, Blaker, Lefevre, Moore, & Krebbers, 2014; Stephen, Coetzee, et al., 2009; Stephen et al., 2011; Stephen, Law Smith, et al., 2009; Tan & Stephen, 2013), or included lips in the colour transformation but again, maintained the same uniform colour change across the face (Stephen, Coetzee, et al., 2009). Although, one study has demonstrated lip colour plays a role in judgements of attractiveness (Stephen & McKeeganh, 2010). Stephen and McKeeganh found that both increased values of lip redness and yellowness were deemed to be more attractive but these effects were greater in female faces. Another recent study investigated location specific colour changes on perceived health and found that redness and lightness were associated with judgements of perceived health only in the cheeks and periorbital regions respectively and not vice versa (Jones, Sweda, Porcheron, & Russell, 2016). Findings in the cheeks are consistent with ours showing that experimental illness reduced redness.

Here, we used the natural colour information in faces associated with experimentally induced illness in Study 6 to test whether this information can reliably be used to inform accurate judgements of health. We also isolate two key facial regions which show the largest changes in colouration: cheeks (which showed highest measured change in skin redness) and lips (which showed a marked change in colouration in averaged images) to test where in the face colour changes are most influential in terms of health judgements. Variation in expression or mood was controlled for on trials of whole faces by blacking out eye and lip regions.

If colour changes recorded in Study 6 are perceptible and used accurately to inform judgements of health, we can expect participants to judge images taken in the placebo condition as more healthy that those taken in the LPS condition.

5.4.2. Methods

5.4.2.1. Design

A forced choice experimental design was employed to determine whether colour changes in the face can accurately inform judgements of health. Identity matched images (for LPS and placebo conditions) were presented over 3 conditions, displayed as: whole faces (with eyes and mouth obscured); isolated cheek patches; and isolated lips.

5.4.2.2. Stimuli

Photographs taken at 2 hours post injection during Study 6 were used in the present study (See section 5.2.2.3)

Images were manipulated to create 3 conditions in which colour from specific facial regions were isolated. To maintain maximum colour information across the face but control for variation in expression, eyes and lips were blacked out. These images were used in a block of trials hereafter referred to as whole faces.

For a second block, showing colour change in the cheek only, patches were cut from each image using *Psychomorph* software, facial images were first aligned by eyes so that the patch was taken from the same location in each image.

Finally, for a third condition, lips were isolated using *Psychomorph*. Lips were first morphed to the average shape for each identity before being isolated so that LPS and placebo lips would differ only in colour and not shape. Examples of stimuli from each condition can be viewed in Figure 18.

Figure 18: Example stimuli from each condition of perceptual experiment.

5.4.2.3. Participants

Forty-six participants (31 female, 14 male) took part online and were recruited through the perception lab webpage. Thirty-nine individuals reported being of Caucasian ethnicity, two reported Chinese ethnicity, two reported Hispanic ethnicity, one reported "other", and one declined to answer. Due to an oversight in coding, participants aged 40 or over were not able to respond with a specific age and instead were bracketed together as ≥40. This affected eight participants. Remaining participants ranged in age from 18-35 (mean 25.29 SD 5.03)

5.4.2.4. Procedure

Over three conditions, each with 22 trials, participants were shown pairs of images. On each trial, images were identity matched showing the same individual two hours post LPS or placebo injection. On each trial participants were asked to click on the image that looked healthiest. Presentation of stimuli was randomised.

Before beginning the experiment participants gave informed consent and were greeted with the following text:

"Can you tell from a face whether someone is ill? What about just part of the face? For this experiment, volunteers had their photo taken twice, once when ill and once when well. In three blocks you will see pairs of images; these images will be of either our volunteers lips, patches taken from their cheeks, or their whole face but with eyes and lips blacked out. Your task is to click on the image in each pair which you think looks healthiest"

Results were recorded as the percentage of trials in each condition which participants selected the placebo image as healthier.

5.4.2.5. Statistical analysis

For each condition, results were subjected to a one-sampled t-test to test whether observed results varied significantly from chance (i.e. 50%). Kolmogorov-Smirnov confirmed that all data from all conditions were normally distributed ($p>.05$)

Conditions (whole faces, cheeks, lips) were then compared with a repeated ANOVA and bonferroni pairwise comparisons to test whether participants were more accurate in their judgements of health in one or more conditions. Mauchly's test confirmed that the assumption of sphericity had not been violated ($p>.05$)

5.4.3. Results

Data showing the average performance of participants in each condition is shown in Figure 19. One sample t-tests confirmed that performance was above chance in all conditions i.e. participants were able to select the "healthy" placebo image in the majority of trials for whole faces ($t(44)=5.49$, $p<.001$), cheeks ($t(44)=2.56$, $p=.014$), and lips ($t(44)=9.76$, $p<.001$).

Performance also varied by condition $F(2,88)=14.20$, $p<.001$, $\eta^2_p=.346$ with performance in the lips condition significantly higher that than in trials containing isolated cheek patches (*Mean difference* =13.64%, *SE* =2.86, $p<.001$) and whole faces (*Mean difference* =8.69%, *SE* =2.50, $p=.004$). Performance did not differ between isolated cheek patches and whole faces (*Mean difference* =4.95%, *SE* =2.39, $p=.132$).

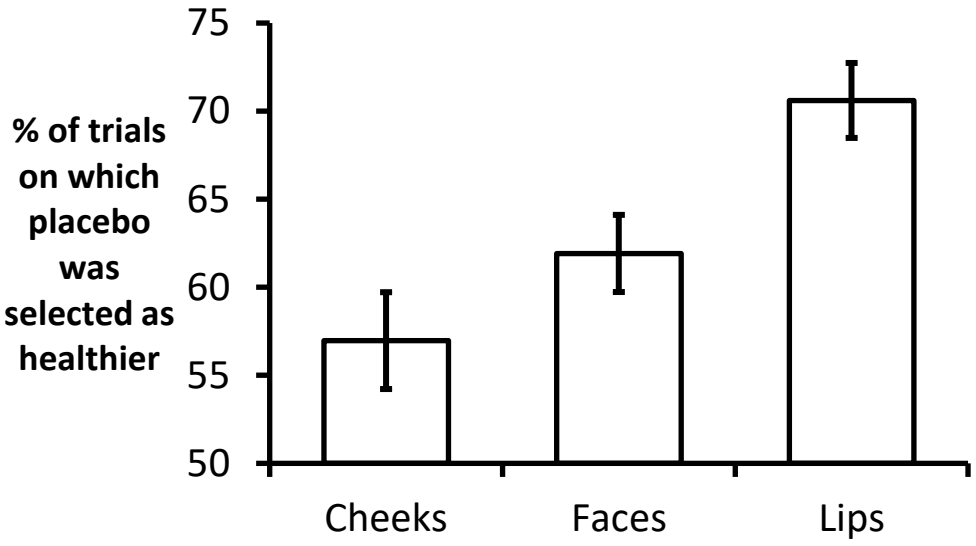

Figure 19: Proportion of trials on which placebo image was selected as healthier than LPS image. Performance on all three conditions was significantly above chance and performance on lips condition was significantly higher than others. Error bars show 95% confidence intervals around individual means.

5.4.4. Discussion

Results of the perceptual study confirm that skin colour change associated with an acute inflammatory response is detectable and can be used to reliably inform judgements of health. Participants were able to correctly identify the healthy image from each pair at a level significantly above chance when presented either with isolated patches of cheek skin, isolated lips, and whole faces with mouths and lips obscured.

Performance on the lips trials was significantly higher than that on the other two blocks, suggesting that colour change here with illness is particularly informative of health status. Lip skin is thin and highly perfused with blood. Given that the changes in skin colour noted in Study 6 are believed to be explained best as changes in blood perfusion and oxygenation, it is not surprising that performance was best in this condition. This study is however the first empirical demonstration that lip colour is used to accurately inform judgments of health and is used more accurately than colour elsewhere in the face. Findings are supported by work of other demonstrating that the contrast between lips and surrounding skin influences perceptions of health (Russel, Porcheron, Sweda, Mauger, & Morizot, 2015; Scott et al., 2010). A particular strength of this study is that lip colour was isolated and judgments were uninfluenced by potential change in lip shape that would have been apparent in original images.

There was no significant difference in performance on trials containing isolated cheek patches and whole faces (with eyes and lips obscured). This may be surprising as whole faces contain cheek information but also additional and relevant colour information. Given that colour change was apparent across the whole face from averaged images, it could be anticipated that this additional colour information would have improved accuracy of health judgements. The lack of difference in performance between cheek and whole face trials could be because the contrast between skin colour relative to lips or sclera inform judgements of health (Russel et al., 2015; Russell, Sweda, Porcheron, & Mauger, 2014); obscuring lips and eyes could have deprived participants of important references points for perceiving contrast. The lack of improved judgements with whole faces relative to cheeks could also be a result of additional distracting information (for example, different hairstyles on each day). The findings, which show no difference in performance between cheeks and whole faces are however, consistent with work showing that judgements of health made from cheek patches correlate with those from whole faces (Jones, Little, Burt, & Perrett, 2004)

A limitation of the present study was the crude nature of controlling for expression in whole faces. Whilst blacking out the eyes and mouth of raw images was effective in removing cues to mood or arousal states, some other, potentially relevant colour information was lost, primarily around the periorbital region. Changes in lightness here have been shown to influence judgements of health (Jones et al., 2016). Controlling for expression in order to investigate the perceptual effect of skin colour (as intended) is difficult in 2D images as shape is portrayed by variation in colour information. Morphing facial images to the average shape

of each identity (as was done for lips trials) would have been an alternative way to control for expression whilst retaining periorbital and lip colour information. However, this method has its own limitation and some detail in colour information would have been lost or distorted as pixels were stretched. Future work should be advanced in two directions; it will be important to isolate colour information from the periorbital region and compare accuracy of health judgements from this area relative to other areas (for example lips and cheeks). Jones et al (2016) for example found that increased cheek redness and periorbital lightness had equal benefits to perceived health; yet a reduction in periorbital lightness had a great negative influence on perceived health than decreasing cheek redness. It would also be informative to investigate how information from skin colour and expression are combined to inform health judgements.

A further limitation of the current study is the presence of hair in whole face images. This limitation is minor as unlike variation in expression which would have been systematically affected by the treatment; participants styled their hair before arrival and were blinded to condition. Therefore, variation in hair across trials can be considered random variation and although it may have provided distracting information for judgments of health, it would have increased the ecological validity of whole face trials.

In summary, this study demonstrated that colour information from faces and facial regions can be used accurately to inform judgements of health status. Furthermore, the lips are particularly informative and well utilised in health judgements.

5.5. Chapter summary and conclusions

Together, studies 6, and 7 demonstrate that the acute inflammatory response is associated with a change in skin colouration which is both detectable and utilised in terms of health judgements. The innate inflammatory response is one of the first lines of defence mounted by the immune system and so will be relevant in most cases of sudden onset illness caused by bacterial or viral infection (Beutler, 2009). Being able to detect such cues could form part of a behavioural immune response and would have been evolutionary advantageous in aiding healthy individuals to avoid infection through contact with sick individuals.

The skin colour change noted with the inflammatory response included a reduction in yellowness as predicted but contrary to predictions, this change was not concurrent with a

reduction in skin carotenoids (which occurred after skin yellowness had recovered). Although skin carotenoid colour was unaffected in response to immune stimulation, a reduction in plasma carotenoids was noted 7 hours post injection. This late reduction in plasma carotenoids could lead to a delayed reduction in skin colouration and should be investigated in future work. It is also probable that prolonged or more severe illness would eventually be reflected in skin colourations as it was found that baseline skin yellowness correlated highly with baseline plasma carotenoids.

The reduction in skin yellowness and more notable drop in skin redness measured in Study 6 are best explained in terms of changes in peripheral blood perfusion and oxygenation status. The skin colour change peaked early in the inflammatory response (1-2 hours post injection) around the same time as peak cytokine production and ahead of peak body temperature (3 hours post injection). Colour change could be a by-product of the body's attempt to conserve heat in the early stages of fever production and could prove valuable in predicting impending severity of illness. Although there was a general reduction in skin redness and yellowness, the form and magnitude of colour change varied topographically. Measured change was greatest in the palm and the cheeks although averages of photographs revealed that the lips also showed a pronounced change in skin colour.

The colour change observed in response to LPS provided a reliable cue to health status. In Study 7, independent raters were able to identify the healthy placebo images on pair matched trials at a rate significantly above chance. The lips were identified as a particular facial region that provided the most reliable colour cue to inform judgments of health.

This series of experiments was unique in demonstrating that skin colour changes with acute activation of the immune system and that the colour change in its natural form is associated with accurate judgements of health. Carotenoid colouration of human skin is not immediately affected by acute activation of the immune system but skin yellowness does reflect basal plasma carotenoids which show delayed depletion with acute inflammation.

Chapter 6: General discussion

6.1. Summary

Taken together, the findings of this thesis provide evidence that natural variation in the carotenoid content of human skin can act as a cue to health. The literature review presented in Chapter 1 highlighted the prominent role that carotenoids play in sexual signalling of non-primate species, and gave precedent for predicting that they may also act as a cue to health in humans. This precedent was based largely on a body of evidence showing that carotenoids measurably influence the yellow appearance of skin and that yellowness is perceived as attractive and healthy looking. Such evidence was based largely on studies in Caucasian populations and although there was abundant evidence that variation in carotenoid colouration could in theory influence judgements of health across varying skin tones, this had not been experimentally demonstrated in a self-contain study.

Chapter 2 of this thesis addressed that very limitation of the literature, presenting an analysis of colour change with increased carotenoid consumption in a sample of10 individuals with a range of constitutive skin tones. Facial photographs of participants before and after the carotenoid intervention were also subjected to a forced choice test of apparent health. Consistent with prior work, results confirmed that increased fruit and vegetable (and hence carotenoid) consumption leads to a measurable increase in skin yellowness within 4 weeks. When a median split by constitutive skin lightness was performed it was found that skin yellowness increased to the same degree in both groups. Further, it was found that the naturally obtained colour change observed in response to two extra portions of vegetables per day was sufficient to influence judgements of health. This finding was small in effect size but highly significant and robust as it was found to be of the same magnitude regardless of whether stimuli were presented as natural un-manipulated images or with the colour change isolated and applied to images which differed in no other dimension. Findings from Chapter 2 are supported by studies showing skin colour changes with carotenoid consumption in populations of varying skin pigmentation (Coetzee & Perrett, 2014; Pezdirc et al., 2015; Stephen et al., 2011; Tan et al., 2015; Whitehead, Re, et al., 2012); and others showing a preference for skin yellowness or carotenoid colouration (Coetzee et al., 2012; Lefevre & Perrett, 2014; Whitehead, Ozakinci, et al., 2012; Whitehead, Re, et al., 2012). This chapter brings together these two lines of evidence providing a demonstration that amongst individuals of varying skin pigmentation, the colour change associated with a modest change carotenoid consumption is sufficient to favourably influence judgements of health.

Literature reviewed in Chapter 1 highlighted that carotenoid colouration of ornaments in non-primate species reflects more than carotenoid availability. These ornaments have been shown to reflect various aspects of health although specific mechanisms underlying these signals are not fully understood. Following confirmation that a modest change in diet was reflected in skin colour to an extent that influenced perceptions of health; Chapters 3 through 5 went on to explore how variation in skin yellowness between and within individuals related to various aspects of past or current health in a bid to investigate whether carotenoid colouration in human skin reflected more than carotenoid consumption.

Chapter 3 investigated several risk factors for health which were thought to be relevant to overall "condition" of an individual. In two studies, skin yellowness was assessed in relation to smoker status, exercise habits, fruit and vegetable consumption, alcohol intake, psychological stress and body fat between individuals (Study 3), and in response to change in these variables within individuals (Study 4). Across both studies, only stress showed a relationship to skin yellowness which was independent of fruit and vegetable consumption. Individuals who reported higher levels of psychological stress were found to have less yellow skin, and within individuals, increases in stress were associated with concurrent decreases in skin yellowness. In both instances, skin lightness or change in skin lightness was unrelated to stress and so the relationship is best explained by variation in skin carotenoids and not melanin content. Other aspects of health which were predicted to be reflected in skin colouration showed no such effect. This may have been because the health relevant variables measured are not the best indicators of current condition in our sample of young health adults. Condition refers to an individual's current ability to maintain optimal functionality, encompassing somatic state, genotype and epigenetics (Hill, 2011). In Chapter 3, health relevant variables were measured which are known to be risk factors for health, such behaviours undoubtedly have long term and delayed implications but it is possible that psychological stress was the only variable affecting *current* condition.

In Chapter 4, skin yellowness was investigated in relation to signs and symptoms of common infectious diseases. It was found that individuals who reported more frequent and severe symptoms of ill health in the prior two months, had lower levels of skin yellowness. Furthermore, when a subsample of participants returned for a follow-up eight weeks later, those who scored higher in terms of retrospective symptoms of illness (relative to baseline) showed a decrease in skin yellowness whilst those scoring lower showed an increase in yellowness. When yellowness was found to be related to retrospective symptoms, there was

no concurrent change in skin lightness. This shows that results are not well explained by melanin and more likely reflect skin carotenoids. Despite changes in retrospective symptoms being reflected in skin yellowness, it was not the case that current health status was associated with changes in yellowness. When participants reported feeling currently ill (during the week prior to testing), they were no less yellow than when they reported not feeling ill. Between individuals, skin yellowness was found only to predict retrospective symptoms from the previous eight weeks when measured at the inner forearm, but within individuals, *changes* in retrospective symptoms were reflected in both the palm and inner forearm. Carotenoids accumulate quickly in the palm, hastened by a high density of sweat ducts but are lost slowly here as skin is thick and turnover is slow (Stahl et al., 1998). The majority of returning participants scored lower in terms of prior symptoms on the second visit. It is thought that the resultant increase in available dietary carotenoids (those not expended with illness) was reflected quickly in the palm skin. A decrease associated with ill health would take longer to show here and eight weeks may not have provided a sufficient time scale to reliably discern retrospective symptoms *between* individuals in Study 5a. This logic would suggest that whilst sweaty regions will reflect short term gains in carotenoid availability, less sweaty regions will provide a more reliable reflection of healthy history. Studies in birds and fish generally measure differences in carotenoid ornaments weeks after infection, or after a feather moult (Brawner et al., 2000; Faivre et al., 2003; Hill et al., 2004); and those testing within two days have found no change in carotenoid colour of ornaments (Koutsos et al., 2003; Perez-Rodriguez et al., 2008). These timescales of effects are consistent with our own and suggest that carotenoid colouration of skin reflects the timescale in which it takes carotenoids to reach the skin. Interestingly, species which display carotenoid signals (e.g fish, bird), don't sweat; and this may be an important constraint in maintaining the honesty of a signal which reflects prior health.

The relationship between current health and skin yellowness was further explored in Chapter 5 using colour data measured during an experimentally induced model of sickness. In a randomised cross over design, twenty two participants received an injection of saline placebo or LPS which is known to illicit a temporary innate immune response, mimicking bacterial infection. Several important findings emerged from this study with respect to skin carotenoids as a cue to health. Firstly, it was found that baseline plasma carotenoids levels were significantly correlated with skin yellowness. Secondly, plasma carotenoids fell in response to LPS during the course of the experiment. Despite these first two findings, skin

yellowness was not found to change with the observed drop in plasma carotenoids, and so this would suggest that immediate changes in carotenoid status are not reflected in carotenoid colouration of human skin. This is consistent with findings from Chapter 4 which found no relationship between current health status and skin yellowness. Nevertheless, baseline correlations between plasma carotenoids and skin yellowness, together with findings from Chapter 4 that skin yellowness relates to retrospective symptoms, suggest that a further or prolonged decrease in plasma carotenoids (from persistent illness) is likely to be reflected in skin colouration.

Skin yellowness was found to change in response to experimentally induced illness, just not in a manner that was consistent with an interpretation of changing carotenoid levels. Skin yellowness fell significantly in the first two hours post injection together with a larger decrease in skin redness, but later recovered whilst plasma carotenoids fell. The change in yellowness was thought to be best explained by changes in blood perfusion and or oxygen saturation and a follow up perceptual experiment demonstrated that the colour changes recorded two hours post injection were used to accurately inform judgements of health. This finding is important because it highlights that other, non-carotenoid related changes in skin yellowness are both relevant to health status and likely to guide judgements of health.

Overall, evidence from this thesis supports the notion that carotenoid colouration of human skin reflects recent health status, and is likely to reliably inform judgements of health. Skin yellowness is related to plasma carotenoid levels and changes in response to modest dietary changes in individuals with a range of constitutive skin pigmentation. Chapter 3 demonstrated that psychological stress in addition to carotenoid consumption will influence skin yellowness, but that other risk factors arguably more relevant to future health outcomes were not reflected. Studies from Chapters 4 and 5 suggest that the health relevant information portrayed in skin yellowness most probably reflects the timescale related to skin cell turn over; but that sweaty regions will provide a more relevant reflection of more recent carotenoid consumption.

6.2. Theoretical contributions, considerations and limitations

6.2.1. Carotenoids cue health

In understanding the evolutionary relevance of a preference for skin yellowness, the work contained in this thesis suggested that skin yellowness reflects more than carotenoid

consumption, and that skin colouration from carotenoids can act as a cue to the bearer's health.

Findings from Chapter 5 showed that whilst skin yellowness reflected participants' health in the prior eight weeks, it was not reliable in indicating current health status (or that of the prior week). This idea, that skin carotenoids do not reflect more immediate changes in ill health was supported by findings from Chapter 5 which showed no clear evidence of skin colour changes consistent with carotenoid loss. Chapter 5 was instrumental however in providing evidence that skin yellowness reflected plasma carotenoids at baseline. Findings from the two chapters together provide convincing evidence that lasting changes in carotenoid availability will be reflected in skin colour, reflecting health history of an individual on a scale of weeks to months.

Findings from Chapter 3 suggest that carotenoid colouration is more relevant to recent experiences than risk factors that are likely to affect future health outcomes. There was no evidence that carotenoid colouration reflected smoker status, body fat, alcohol consumption or exercise habits, but there was evidence of psychological stress in the prior week relating to skin yellowness. Together with findings from Chapter 4, showing symptoms of ill health in the prior two months relate to skin yellowness, this would suggest that there are direct evolutionary benefits to be gained from a preference for skin yellowness e.g. avoiding contagious illness today. Although it remains to be tested whether indirect benefits, in terms of selecting for good genes for health, are to be gained also.

6.2.2. Carotenoids and colour

Throughout this thesis, carotenoid content of skin was inferred from skin colour rather than measured directly. With respect to understanding the evolutionary significance of a preference for yellowness, and whether it is linked to carotenoids (which was the aim of this thesis), the inference of carotenoids from colour measures is not a limitation. In fact, testing whether skin yellowness related to health was necessary to answer the question of whether skin carotenoids act as a cue to health beyond diet, because judgements of health are based upon the appearance of skin colour. Assessing skin colour in terms of CIE L* a* b* space allowed empirical measurements to be taken which directly relate to the visual appearance of skin colour. It is acknowledged that melanin and blood perfusion or oxygenation status can influence the yellow appearance of skin, but these pigments also contribute to changes in lightness and redness. Consequently, throughout the experimental work presented, any

associations found between health and skin yellowness were also explored with reference to these other colour axes. Doing so allowed an inference to be made that the variation in yellowness associated with health in Chapters 3 (stress) and 4 (health history), were most likely to be driven by variation in carotenoid content of skin. Chapter 5, was instrumental in demonstrating that variation in skin yellowness with illness is not always best explained by carotenoids. In Study 6, a drop in blood perfusion or oxygenation more adequately accounted for a drop in skin yellowness witnessed. Such a finding does not compromise the validity of carotenoids as a cue to health because with a shift in oxygenation status of blood, the skin also becomes darker and less red; the colour of blood status will therefore be visibly different from that of carotenoid status. Furthermore, the relationships between blood oxygenation, skin yellowness and health are all positive so contribute in the same direction towards an association between increased yellowness and increased perceived health. The knowledge that other chromophores contribute to skin yellowness, does not then threaten the interpretation that variation in carotenoid content may act as a cue to health. What this acknowledgment does suggest though is that colour measurements will not be the most accurate way to determine carotenoid levels, and this will add limitations to some of the practical applications arising from the finding that skin carotenoid content is related to health status (see below, section 6.3)

6.2.3. Why are we not bright yellow?

If carotenoid colouration of human skin is a valid reflection of health status, and is deemed more attractive by others, it is reasonable to ask why we are not brightly coloured, as are carotenoid ornaments of many birds and fish.

Within evolutionary biology there is a distinction between cues and signals (as noted in the introductory chapter: 1.4). Cues are traits (such as skin colour) that are assessed during mate choice and influence the mating decision. If this trait is modified to serve a purpose of communicating mate quality to others, it becomes a signal (Candolin, 2003). Often a trait will be exaggerated to a point where it becomes detrimental to survival but increases attractiveness to the opposite sex. Bright feathers and fins for example will increase predation risk; and carotenoids displayed in feathers will become unavailable for physiological functions, but bright ornaments will increase mating success and therefore there is an evolutionary pressure for them to continue to become brighter. Cues, meanwhile can be maintained due to other selection pressures, carotenoid colouration of human skin for

example will be maintained as a cue simply because consumption of carotenoids afford physiological benefits and excess carotenoids will passively colour the skin.

If carotenoid colouration had evolved as a signal to communicate health status we may expect variation in colour to be greatest in body locations most readily observable (e.g. the face). Here, as in other studies (Stahl et al., 1998; Alaluf, Heinrich, et al., 2002) we find greatest variation in yellowness at sweaty regions (e.g. palm and forehead). This would suggest carotenoid colour evolved simply as a potential cue that arose as a by-product of physiological processes.

Perceptual studies have demonstrated that there is a limit to the degree of yellowness which others find attractive or healthy in appearance. In interactive trials, when given the opportunity to optimise apparent health by digitally altering skin colour; participants reliably add yellowness or carotenoid colour to stimuli, but they do not apply the maximum available colour (Stephen et al., 2011; Whitehead et al., 2013). There is a limit then to the positive relationship between skin yellowness and perceived health. This may be constrained by a negative selection pressure associated with a high degree of skin yellowness.

Jaundice is a yellowing of the skin, sclera and mucosa that occurs as with a build-up of bilirubin: a breakdown product of blood cells. Spectrophotometrically the colour of bilirubin is very similar to beta-carotene and in normal healthy individuals, bilirubin acts as an endogenous antioxidant, serving a similar role as carotenoids in the maintenance of health (Neuzil & Stocker, 1994). At normal levels then, bilirubin is unlikely to compromise the validity of the relationship between skin yellowness and health. Jaundice present only when there are abnormally high levels of bilirubin and in adults can be a sign of serious health complications such as liver dysfunction, pancreatitis and biliary tract infection (Roche & Kobos, 2004). This association between very high levels of skin yellowness and serious health problems, could reasonably act as a negative selection pressure for ever increasing skin yellowness. This negative selection pressure would have prevented carotenoid colouration of human skin from evolving from a cue to a signal of health.

It is important to note that although participants will not maximise yellowness to optimise apparent health on interactive trials; in studies of natural images, a linear relationship does exist between skin yellowness and apparent health (Henderson et al., 2016; Scott et al., 2010; Stephen et al., 2012). Such studies are invariably conducted with stimuli of young healthy adults where examples of extreme yellowness associated with jaundice is not present. Given

that the studies within the present thesis were also restricted to young and healthy samples, the use of linear statistics throughout was deemed appropriate.

6.3. Practical implications

6.3.1. Screening and monitoring

Measuring skin carotenoids using light spectroscopy methods has been touted as a tool for assessing overall antioxidant status of individuals for monitoring health (Lademann et al., 2014) and a commercial device has been launched which claims as much[3]. Much of the evidence underpinning claims that measurements from this device, or from Raman spectroscopy methods, reflect stress (Lademann et al., 2014), illness (Darvin et al., 2010), or intense exercise (Vierck et al., 2012) is still in its infancy, with small samples of participants and predominantly descriptive statistics (See Introduction Chapter Section 1.5).This thesis however, provides support that measurements of skin carotenoids really could be informative of health status and may be useful in screening individuals for recent illness, or stress.

Chapter 3 demonstrated that psychological stress is reflected in skin carotenoid colouration, and Chapter 4 demonstrated that carotenoid colour of skin was linked to symptoms of illness in the prior eight weeks. Psychological stress increases vulnerability to infection (Cohen et al., 1991), and those who are ill often in the past may be more likely to become ill in the future. Carotenoid content of skin could therefore potentially have applications as a medical tool, employed to screen for vaccine priority or asses risk associated with surgical intervention. It could also be used by individuals as a tool for monitoring some global measure of healthy living.

Such applications are theoretically supported by the work contained here but are still far from realisation. Whilst Chapters 3 and 4 demonstrated that carotenoid colouration reflects aspects of health beyond diet, Chapter 5 demonstrated that skin yellowness can change with illness through other mechanisms also. Measuring skin carotenoids specifically (possibly using Raman spectroscopy), as opposed to skin colour will be more relevant to any monitoring or screening applications but further work will be necessary in validating such methods. Validation should include confirmation of whether Raman methods are detecting carotenoids

[3] http://mybiozoom.com/en/home/

in the plasma as well as those in the skin, since plasma and skin levels will respond on different timescales (as seen in Chapter 5) and therefore may reflect different information.

In moving forward with the potential application of non-invasive carotenoid measurements to provide meaningful information about health, it will be necessary first to determine whether skin carotenoid measures can be isolated from carotenoids elsewhere (i.e. plasma), and also from other pigments present in the skin. Only then can we further explore what meaningful information is reflected in skin carotenoids levels and how this can be applied.

6.3.2. Motivating behaviour change

Harnessing evolutionary theory, by appealing to our sense of vanity has been suggested as a means of motivating positive behaviour change (Whitehead et al., 2013; Whitehead, Ozakinci, et al., 2012). Indeed, in a randomised trial, it has been shown that a personalised intervention, demonstrating the appearance benefits to be gained from an increase in fruit and vegetable consumption was more motivating than written information describing health benefits of a diet rich in fruit and vegetables (Whitehead et al., 2013). Whitehead and colleagues found that 10 weeks after being allowed to choose the intensity of carotenoid colouration which participants believed they looked most healthy with, and given a copy of this image to take home, participants had sustained an increased level of fruit and vegetable consumption. This was not true of the comparison group who were given written health promotional material. This study suggests that we are motivated by short term gains in apparent health. Findings of the present thesis reveal that the skin colour transform which motivated improvement in dietary habits, is not only influenced by carotenoid consumption but also by stress (Chapter 3) and instances of illness (Chapter 4). This new understanding could be incorporated into appearance based interventions. By explaining that carotenoid colouration reflects aspects of health beyond diet, individuals may be motivated not just to increase fruit and vegetable consumption, but to take a more holistic approach to health care, for example stress management.

6.3.3. Evaluating health

Studies have shown that assessments of health made by observers can predict health outcomes (in terms of disease burden or mortality risk) of others, to a greater extent than a review of past or current health conditions. For example, Doorn (1998) found that wives' estimates of the likelihood that their husbands' would live 10 years was able to predict 3-year mortality. This was true even when other recorded measures of health were controlled,

including, the husbands' past hospitalisations, smoking history, current health behaviours, physical problems, medications, and self-ratings of health. Wives of course will be intimately familiar their husbands health but a study by Brissette, Leventhal and Leventhal (2003) has shown also that health judgements of independent observers (trained research assistants) were able to better predict disease burden and future mortality risk than self-rated health, or the linear combination of all information recorded during a medical history review of 70 diseases.

Such studies show that in terms of assessing health there is value in human interaction; that observers may be capable of capturing information about the health status of targets that would be missed by a written review of health conditions. At this point is not known what information observers utilise to make judgments of health (although this may include observations of how the individual, looks, smells, sounds or moves), it is also not known how these are combined. Whilst this requires further research, findings from study 1b and study 7 of this thesis demonstrate that valid judgements of health can be drawn from faces and more specifically, from skin tone (see also Appendix 1 for further review of facial cues to health and their validity). Skin colouration may therefore play a part in the predictive value of health assessments made by observers. Future work should investigate how observers incorporate different sources of information to make a global assessment of health.

6.4. Future directions

6.4.1. Exercise

In Chapter 3, exercise was a surprising null effect with relation to carotenoid colouration of skin. Many other health relevant variables were explored in relation to carotenoid colouration of skin and most were found to show no relationship, including body fat, smoker status, alcohol consumption, and adiposity. Upon reflection of these findings, it was thought that many of these risk factors predict long term consequences and future chronic disease whereas psychological stress is likely to affect short-term health outcome, leaving individuals more vulnerable to the common cold for example. If carotenoid colouration of human skin is reflecting recent experiences of illness (as suggested by Chapter 4) then this distinction between short-term and long-term risks to health would explain most of the findings from Chapter 3. Regular exercise however, like stress, has been linked to reduced susceptibility to the common cold (Nieman et al., 2011). The lack of effect between exercise and skin colour is therefore hard to explain unless there was insufficient variation in exercise habits between

our participants or the measures employed were inadequate Future work should therefore further explore the relation between exercise, fitness and skin colour to confirm or deny whether carotenoid colouration of skin reflects lifestyle variables related to short-term health outcomes. Similarly, quantity and quality of sleep is known to affect vulnerability to infections (Cohen, Doyle, Alper, Janicki-Deverts, & Turner, 2009) and may be a prosperous avenue of research for further understanding the information content of skin carotenoid levels.

6.4.2. Predicting future health outcomes

Another informative avenue of research will be to test prospectively, whether carotenoid content or colouration of skin is predictive of future health status. Studies in birds have shown that the colour of carotenoid based ornaments is informative of ability to mount a strong immune response when challenged (Aguilera & Amat, 2007; Blount et al., 2003; Peters et al., 2004) or survive an epidemic (Nolan et al., 1998). In realising any medical based screening application of carotenoid measurements it will be necessary to confirm whether in humans too, carotenoid content of skin is relevant to predicting response to an immune challenge or susceptibility to infection.

6.4.3. Carotenoids, health and constitutive skin colour

Finally, much like the early work demonstrating that carotenoids colour skin and influence judgements, the empirical work presented in Chapters 3-5 has been conducted almost exclusively amongst lightly pigmented, Northern European participants. These were early investigations into the relationship between carotenoid colouration and health beyond diet. As such it was necessary to limit potential confounds from variation in melanin pigmentation. Chapter 2 provided a demonstration that a colour change in response to a modest change in carotenoid consumption was both measurable and preferable in a sample of individuals varying in constitutive skin pigmentation. This provides evidence to suggest that natural variation in carotenoid colouration of skin could act as a cue to health across a range of skin tones. In Study 3 (Chapter 3), the effect size of stress upon skin yellowness was of similar magnitude to that of fruit and vegetable consumption and so both effects of diet and stress on skin colour should be equally visible in human skin. There is little reason to suspect that health effects demonstrated in this thesis should not therefore be reflected in carotenoid colouration of skin types represented in Chapter 2. This assumption, however, awaits empirical investigation, as does an investigation of health in skin tones darker than those represented in Chapter 2.

6.5. Conclusions

In conclusion, this thesis has demonstrated that carotenoid colouration of human skin reflects aspects of health beyond diet. Evidence was presented which demonstrated that carotenoid colouration of skin reflected recent levels of psychological stress and recent instances of ill-health. Both of these effects were demonstrated with reference to variation in carotenoid colouration between individuals and changing levels of carotenoid colouration within individuals, suggesting that the effects are robust. Other health relevant variables were not found to relate to carotenoid colouration of skin and so further work is necessary to unpack the full relevance of carotenoid cues to health and the mechanisms sustaining relationships between carotenoid colouration and health variables which they reflect. Theoretically, this thesis supports the hypothesis that a preference for carotenoid colouration of skin would confer evolutionary benefits by providing cues to the bearer's health and has practical implications in terms of monitoring health and motivating behaviour change.

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Appendix 1: Perception of Health from Facial Cues

The following manuscript was invited for publication in a peer-reviewed journal (accepted December 2015):

Henderson, A. J., Holzleitner, I. J., Talamas, S. N., & Perrett, D. I. (2016). Perception of health from facial cues. *Phil. Trans. R. Soc. B, 371*(1693), 20150380.

Summary

Impressions of health are integral to social interactions, yet poorly understood. A review of the literature reveals multiple facial characteristics that potentially act as cues to health judgements. The cues vary in their stability across time: structural shape cues including symmetry and sexual dimorphism alter slowly across the life span and have been found to have weak links to actual health, but show inconsistent effects on perceived health. Facial adiposity changes over a medium time course and is associated with both perceived and actual health. Skin colour alters over a short time and has strong effects on perceived health, yet links to health outcomes have barely been evaluated. Reviewing suggested an additional influence of demeanour as a perceptual cue to health. We therefore investigated the association of health judgements to multiple facial cues measured objectively from 2D and 3D facial images. We found evidence for independent contributions to face shape and skin colour cues to perceived health. Our empirical findings: (a) reinforce the role of skin yellowness; (b) demonstrate the utility of global face shape measures of adiposity and (c) emphasise the role of affect in facial images with nominally neutral expression in impressions of health.

Introduction

Judgement of a person's health based on their facial appearance is a daily occurrence. Understanding how these judgements are informed is important because they feed into other social judgements such as attractiveness [1–3] or leadership ability [4] which is potentially consequential in terms of real-life outcomes, such as dating and or employment outcomes. Other social consequences may stem directly from judgements of health because maintaining a physical distance from those who are perceived to be unhealthy has clear evolutionary benefits through avoidance of contagious disease. Indeed, when cued with disease-relevant images, people tend to rate themselves as less extraverted, and are quicker to make avoidance movements in response to faces [5]. Negative judgements of perceived health may therefore

lead to a risk of social isolation and stigmatisation [6,7]. This outcome is particularly disquieting in modern Western societies where disease is largely non-communicable, and social contact is known to provide health benefits [8]. A clearer understanding of how health attributions are informed and to what extent they are valid may reduce the negative social consequences which can follow.

To this end, a literature search was conducted in OVID, Web of Science and EBSCO (including PsychINFO) using the following search terms: "face" (title) OR "facial" (title) AND "health" (title) OR "perceiv* health" (keyword) AND "cue" (keyword). The search returned 86 unique abstracts, 32 of which were retained after screening for relevance (i.e. papers reporting empirical results and testing for an association between at least one facial cue and either perceived health or actual health outcomes. The search was by no means exhaustive and additional papers were included from reference lists of identified papers. It should be noted that, although perceived attractiveness is often thought to be a proxy for apparent health, some studies have failed to find a relationship between attractiveness and health [9,10]. Therefore, for brevity and clarity, within this review perceptual studies were limited to those specifically investigating perceived health and not attractiveness. Results of the literature search have been grouped into the headings below, under which evidence of both cue use and cue validity is summarised. Cue use refers to the information in a face (for example colour, shape, expression) which influences our perceptions or judgements of how healthy a person looks. Conversely, cue validity refers to whether the facial information is reliably related to the health of a person. For accurate assessment of health from faces, cues would have to be both valid and used.

Men's Facial Masculinity

Male facial masculinity is thought to be a cue to good health and 'good genes' (i.e. genes promoting health). Based on findings from the animal literature, the Immunocompetence Handicap Hypothesis (ICHH, [11]) proposes that male masculinity (including facial masculinity) acts as a handicap display that reliably conveys heritable aspects of good health to potential partners. Masculine facial features develop during puberty under the influence of androgenic steroid sex hormones (e.g., testosterone). As producing and metabolising testosterone is costly (and might lead to higher oxidative stress [12]), the ICHH suggests that individuals with better innate immunity and health can afford to develop more pronounced (i.e. costly) masculine facial features than individuals of poorer health.

In line with this theoretical framework, several studies have found a relationship between male masculinity and perceived health: more masculine male faces are rated as looking healthier [1,13–15] although this is not a universal finding [16]. Studies investigating whether facial masculinity is indeed related to superior actual health are inconclusive [17]. For example, while both Thornhill and Gangestad (2006) [9] and Boothroyd et al. (2013) [1] found that facial masculinity was negatively related to male participants' reported number of prior colds and flu, mixed results were found regarding the relationship of male facial masculinity and length of infections and antibiotic use, and no association with "stomach flu" was found. Rated facial masculinity has been associated with general health measured as a composite score from adolescent medical records [10] and to one aspect of immune function [18]. Rantala and colleagues found that men with more masculine-looking faces were able to mount a stronger antibody response to a hepatitis B vaccination. Facial masculinity did not, however, mediate the relationship between immune response and facial attractiveness, suggesting that perceived masculinity was not being utilised as a cue to assess mate value.

Facial Symmetry

Facial symmetry is posited to be a cue to health during childhood and development because sources of environmental and genetic stress (e.g., pathogens or mutation rate) would test an individual's ability to maintain developmental stability, and resist asymmetric growth. Only two studies were identified which tested the effect of symmetry on judgements of health and both found a positive relationship [19,20].

Some support for the position that facial symmetry acts as an index to prior health has also been reported. Thornhill and Gangestad (2006) found a positive relationship between facial asymmetry and number of bouts of colds and flu over the previous three years [9]. Facial asymmetry was found to be marginally related to total number of days ill with cold and flu infections, and also with antibiotic use but was unrelated to stomach bugs. Also, Zebrowitz and Rhodes (2004) found that facial symmetry was related to physicians' assessments of health, but this was true only amongst those who scored lower than average with regard to symmetry [14].

Contrary to the hypothesis that symmetry cues health, the largest study of facial asymmetry and health to date found no relationship between these variables [21]. Researchers utilised data from a British cohort study of 4732 individuals and found that facial symmetry at age 15 was unrelated to longitudinal measures of childhood health, including

measures of the proportion of childhood years spent unwell, average number of illness symptoms per year, and total number of infections.

Facial Adiposity

Obesity is known to be related to a number of health risks, and is often indexed by weight, Body Mass Index (BMI, weight scaled for height) or percentage body fat. Recent findings suggest that perceived weight as judged from the face alone, also referred to as facial adiposity, may also provide an important cue to health.

Perceptual studies have demonstrated that facial adiposity in adult faces is related negatively to judgements of health [2,22,23], but positively related to health judgements in infant faces [24]. There is also growing evidence that perceived adiposity from facial images is related to actual health outcomes. For example, individuals that were judged to be heavier-looking reported more frequent and longer lasting colds and had higher blood pressure [22]. Similarly, perceived weight has been found to relate to a composite measure of general condition including items related to physical and psychological health [25]. Indeed, ratings of weight from faces not only predicted weight 36 years later but also predicted adulthood risk of obesity, illness symptoms (muscle aches, shortness of breath and chest pain), chronic conditions (arthritis, high blood pressure and diabetes) and all-cause mortality [26].

In men, perceived facial adiposity has also been linked to the antibody response to a hepatitis C vaccination [18]. This relationship is in accord with findings that hepatitis vaccine reaction is stronger in individuals with lower weight and BMI [27]. In addition, facial adiposity was found to mediate the relationship between strength of hepatitis C antibody response and perceived attractiveness, suggesting that facial fatness is a valid and utilised cue to health [18].

Recently, a surprising finding has emerged: facial cues to body fat may be a better predictor of health outcomes than traditional indices of obesity such as BMI, percentage body fat, or girth of waist. Neck adiposity (measured using a lipometer), for example, has been shown to be a better discriminatory factor in identifying type 2 diabetes than BMI, percentage fat and measurements of subcutaneous fat using the lipometer at 14 other body locations [28]. Similarly, neck circumference has been shown to be a significant predictor of hypertension, independent of BMI and waist circumference [29].

Fat distribution may be more informative than fat mass per se when it comes to health outcomes. If facial fatness provides key information to body fat distribution, this could explain the stronger relationship between perceived fatness from facial images relative to more common indices of body size such as BMI or percentage fat. Indeed, cheek fat is related to visceral abdominal fat [30], which is thought to be a particularly risky place to carry excess weight.

Three quantifiable aspects of face shape have also been identified as influencing judgements of weight and may provide targets for testing their relationship to health outcomes (width-to-height-ratio, perimeter-to-area ratio and check-to-jaw-width ratio) [31]. These three measures do not necessarily capture all aspects of face shape that relate to weight perceived from faces. It is also possible to use an empirical approach to derive more global measures of facial shape that characterise facial adiposity using principal component analysis of landmarks capturing the structure of 2D facial images [32] or the entire surface of 3D faces [33]. It remains to be shown how such measures relate to perceived health and measured health.

Skin Condition (Texture and Colour)

Skin colour and texture are facial cues which are malleable and may change in response to illness in a short space of time. For this reason, it has been argued that such cues should provide more relevant information to current health than shape information such as facial masculinity or symmetry [34].

Studies showing correlations between perceived health in whole faces and isolated patches of skin from those same faces attest to the relevance of skin information in perceptual judgements of health [35,36]. Skin information can be further divided into more specific cues including surface topography (lumps, bumps and wrinkles), colour and colour distribution, each of which have been shown to influence judgements of health (although colour distribution may be more influential than surface topography [37]).

When the colour of faces is manipulated along three axes consistent with human colour perception, i.e. yellowness, redness or lightness, an increase in all three leads to more positive judgements of health [38]. The distribution of colour in the face is also important in judgements of health, with homogenous colour distribution increasing judgements of health compared to patchy colour distribution [39–41]. Contrast in luminance and colour between

the facial skin and features such as eyes and lips also contributes to health judgements [38,42].

In terms of cue validity, it has been demonstrated that judgements of health are more accurate when faces are presented containing relevant skin information alone (and face shape is held constant) compared to when face shape information alone is presented (and skin colour is held constant) [43]. This is consistent with the argument above that skin colour cues (including texture, colour and colour distribution) may provide more health information than face shape (although it should be noted that in [44] colour included shading cues to 3D face shape).

More detailed investigations of specific skin cues and their association to health outcomes are limited within the face perception literature. Several studies have suggested a causal relationship between diets rich in fruits and vegetables [44–48] and skin colour (specifically yellowness), yet there is limited evidence suggesting that this aspect of human skin colouration is related to health beyond diet. There were also no studies identified which tested relationships between measures of health and skin texture or colour distribution as measured from facial images. This is an area of research requiring future attention.

Expression

There is clear lack of experimental studies investigating the role of expression or emotion upon perceived health. One study investigated smiles in photos and their link to health outcomes [49]. Researchers found from photographs of major league baseball players in the United States that those individuals who displayed a full Duchenne smile (with orbicularis muscle activity causing wrinkles around the eyes in addition to zygomatic activity raising mouth corners) were half as likely to die in any given subsequent year, relative to non-smiling individuals. The authors reasoned that individuals who are smiling in a photograph are likely to smile more often in general and that mood is related to physiology and general health. That mood and health are associated is widely accepted [50], and so the prospect that facial cues to mood will influence judgements of health follows logically.

The lack of more empirical studies in this area likely stems from the use of standardised passport style photographs in face perception studies. There is some work that has shown that even amongst standardised photos, in which participants are asked to hold a neutral expression, raters were able to judge images of sleep deprived individuals as more fatigued and sadder than images of the same participants after a normal sleep [51]. Sleep deprived

images were also said to have “droopier mouths” and “hanging eyelids”. Although this study did not investigate perceived health, it does highlight the possibility that variation in expression exists amongst standardised photos, and that raters are able to use this information to inform judgements. Indeed the apparent mood of ‘neutral’ facial poses varies considerably and is the driving force behind many social attributions (e.g., trustworthiness) made to facial images [52]. Further work to explore the use of such cues and their validity in health judgements is necessary.

Summary and Future Directions

Malleable face cues including facial adiposity, skin colour and texture may offer more current and relevant information with regard to health than more stable aspects of facial appearance such as masculinity and symmetry. Indeed, there is some evidence that skin information allows more accurate perceptions of health relative to shape information [43]. Links between actual health and specific skin condition cues (colour and texture) as measured from facial images is currently lacking, although a wealth of evidence attests to the fact that these cues are utilised in health judgements. Expression or mood-related information was also identified as a potentially important cue to health. To date, studies of facial cues to health outcomes have focused on stable structural aspects of the face such as averageness, symmetry and sexual dimorphism. Future research should therefore test the validity of health judgements made in response to malleable facial cues such as colour and resting demeanour.

The adiposity literature revealed the tantalising possibility that facial cues to fatness may predict health outcomes more accurately than traditional measures of body fat or weight, although to date the only evidence supporting this notion comes from studies of neck diameter and neck adiposity measured by lipometer. Given that other facial cues are informing judgements and measures of facial fatness (e.g., chubby cheeks and jaw shape), an important step forward will be to test how measured or rated adiposity from whole faces compares to traditional measures of body weight and size in predicting health outcomes.

Of course, none of these facial cues exist in isolation and, whilst some studies have begun to investigate how cues are integrated to inform judgements of health [20,23,37,39], and the relative validity of cues [14,43], these are two areas of health perception which are still largely unexplored. The expectation may be that multiple cues of health are congruent (as has been argued for cues to mate quality [53–56]).

The Present Study

Here we report results from two empirical studies that we feel are particularly timely in the quest to understand how we use facial cues to assess health. In both studies we test the relative importance of malleable cues in judgements of perceived health. In 3D and 2D images we have objectively measured variation in the upward or downward turn of the mouth as well as the extent to which eyes are opened (hereafter referred to as "mouth curvature" and "eye openness"). These particular facial features were selected for investigation because they are related to perceptions of sadness and fatigue [51] and may therefore also influence judgements of health. In Study 1, the contribution of mouth curvature, eye openness and measured facial adiposity are tested in health judgements of 3D faces. Study 2 tests the relative contribution of these cues together with colour information in health attributions made to 2D facial images.

Methods

All data collection was approved by UTREC and the School of Psychology and Neuroscience Ethics Committee, University of St Andrews. All participants provided informed written consent for their images and data to be used.

Study 1: 3D Images

Stimuli

Facial scans were taken using a 3D camera (http://www.3dMD.com). Participants were 68 Caucasian women (M±SD=20.9±2.4 years, range 18–32) and 50 Caucasian men (M±SD=21.2±2.5 years, range 18–32) who were photographed with a neutral facial expression, their hair pulled back and at a set distance and relative height to the camera [57]Faces were delineated in MorphAnalyser 2.4.0 [58] with 49 landmarks. The landmark templates for all digitized head models were aligned in orientation, rotation and scale using Procrustes superimposition, and surface models were resampled in accordance to a standard head delineated with the same set of landmarks. This process establishes homology of each head model's tessellations across the entire sampled population. Thus, further analyses and

averaging were conducted on the surfaces of the head models as a whole rather than on the template landmarks [33]. Height and weight were measured for all participants and used to calculate BMI. Basic demographic information (age, gender and ethnicity) was also recorded.

Objective measurements of facial stimuli

Facial BMI scores. All head models were subjected to a principal component analysis (PCA). Each head model could then be described with a relatively small number of principal components (PCs). Next two groups were defined, one of 10 individuals low in BMI and one of 10 individuals high in BMI [57]. Due to the sexual dimorphism in body composition and build, BMI-scores were separately calculated for men and women. For men, the average BMI was 19.5 for the low group and 26.9 for the high group; for women, the average BMI was 17.9 for the low group and 28.7 for the high group (See Figure 1). For each of the 118 PCs, the average score of the low subsample was calculated (separately for male and female faces), defining a position in the 118-dimensional space. The average PC scores of the high subsample were similarly calculated. A 'BMI axis' in face space was then defined by the low and high BMI average face shapes. Each face in the sample was projected onto this axis, and the projection value defined the facial BMI score [57]. Average values for each PC were separately calculated for men and women with low and high BMI. Faces in the low and high groups were matched so that low and high BMI groups did not differ in height, ($t(118) \leq .78$, all $p \geq .454$). Facial BMI scores correlated with actual BMI ($r(118)=.59$, $p<.001$), but not height ($r(117)=.05$, $p=.565$) [57].

Eyelid openness. The degree of eyelid openness was examined by taking the vertical distance from the centre of the pupil to the top eyelid and dividing it by the width of the eye inner canthus to outer canthus. This measure was computed for left and right eyes separately and the two values averaged.

Figure 1. Female and male 3D face shapes associated with low (left of each pair) and high (right of each pair) Body Mass Index. For women, the face shapes correspond to BMIs of 18 and 31, respectively; for men, the face shapes correspond to BMIs of 17 and 29.

Mouth curvature. Measurements of mouth curvature were calculated by taking the average height of the right and left corners of the mouth (relative to the base of the image) and subtracting the height of the centre of the mouth (between the lips and directly under the philtrum). This value was then divided by the width of the mouth to standardise the measurement (see [59] for further details and validation of this measurement).

Perceptual ratings

To eliminate the influence of hairstyle, clothing and cues from the neck circumference on perceptual ratings, all 3D heads were masked to show faces only. Average male and female face texture images were created using PsychoMorph 4 [60]. All faces were rendered with this sex-specific standardised texture, so that only face shape differed between each of the 3D face models [33].

Participants residing in the US and of mixed ethnicity were recruited via Amazon Mechanical Turk (46 women, M±SD=37.80±10.71 years, and 70 men, M±SD=33.36±9.09 years). Prior to the rating, participants were presented with static 2D frontal images of all face models to provide an overview of stimulus variability. The 3D face stimuli were then presented in randomised order, 'bobbing' in a sinusoidal manner from left to right and up and down. For each face, participants were asked "Compared to other men/women his/her age, how healthy is this person?" Ratings were given on a 0-100 visual analogue scale. Stimuli were presented

individually against a black background and remained visible until a rating was made. Female and male faces were presented in two separate blocks; the order of blocks was randomised. Ratings were averaged across participants for each face.

Study 2: 2D Images

Stimuli

Facial photographs of 67 Caucasian women (M±SD=20.85±2.15 years, range 18–29) were taken using a camera in D65 lighting in a photographic booth painted with spectrally neutral grey and colour reference card (Gregtag Macbeth Mini ColorChecker Pantone). Clothing was covered with a grey-coloured board to prevent coloured reflection from clothing affecting facial illumination. Participants were photographed with a neutral facial expression, their hair pulled back and at a set distance and relative height to the camera. Faces were delineated in PsychoMorph with 187 landmarks. Face images were aligned on left and right pupils and were cropped to maximise the size of the face within the image frame.

Objective measurements of facial stimuli

Skin Colour. Square image patches were cut from the left and right cheek areas and from the forehead. Average CIE L*, a*, b* colour in each cut patch was computed and the average colour was calculated by averaging across all three patches,

Eyes and Mouth. Measurements of eyelid openness and mouth curvature were calculated in the same manner as described for Study 1.

Facial BMI scores. As detailed for the 3D faces, a data-driven approach was employed to measure facial adiposity. A principal component analysis of face shape determined by 117 of delineation points (including the nose, mouth, eyes, and eyebrows, forehead, chin and neck) was used to define a BMI axis for female faces and to subsequently score individual faces along this axis.

Perceptual Ratings

Participants were recruited and paid via Amazon Mechanical Turk to provide ratings of perceived health (11 females and 21 males, M±SD=40.16±12.44 years). Analysis was restricted to participants who reported the same ethnicity as the images presented, i.e. “white

Caucasian", as sensitivity to skin colour cues may be weaker when raters are asked to make judgements from faces of other ethnicity [61].

Evaluators first previewed all stimuli with each image displayed for one second. The stimuli were then re-presented in random order so that participants could rate each face for perceived health. A minimum viewing time of one second per image was set, but no maximum response time was enforced. Facial ratings were made on a 7-point scale with endpoints "not at all healthy" to "very healthy". Cronbach's alpha for health ratings was high (n=32, α=.94).

Composite facial images

To illustrate the difference in appearance between faces rated high and low for perceived health, composite images were created. Each composite consisted of faces which on average received the 10 lowest or highest ratings of perceived health. Mean ratings were 3.5 and 5.7, respectively. In PsychoMorph, 184 facial landmarks were placed on each image before composites displaying the average shape, colour and texture were created [60].

Results

Study 1: 3D images

Figure 2 displays the relationship between facial BMI scores and perceived health. Curve fitting revealed a significant quadratic relationship (R^2=.25, $F(1,115)$=19.45, $p<.001$) but a non-significant linear relationship (R^2 =.01, $F(1,116)$=0.95, p=.331). Mouth curvature was found to relate to perceived health, ($r(118)$=-.20, p=.030) in that more downturned mouths were associated with lower ratings of perceived health. Eye openness was unrelated to perceived health ($r(118)$=-.02, p=.865).

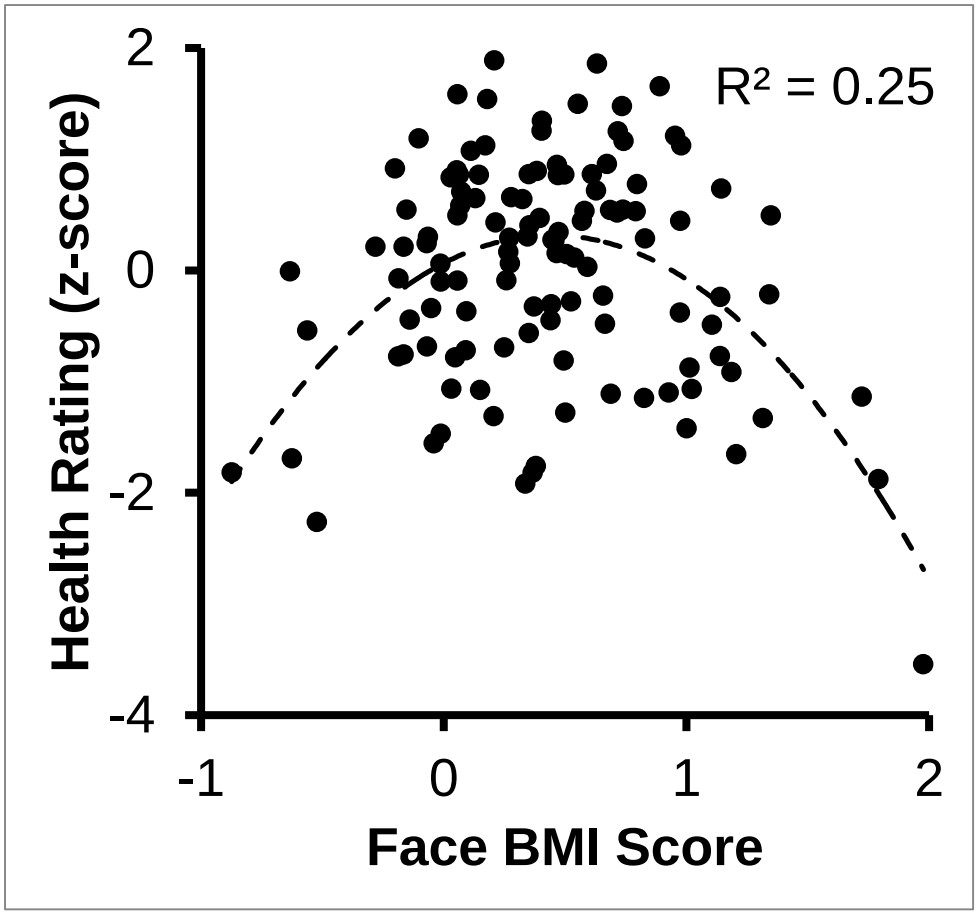

Figure 2. Relation of facial shape and perception of health for a set of 3D head models. The dashed line gives the best fit quadratic function relating perceived health to the face BMI score (variance explained R^2=.25). The quadratic relationship shows that face shapes associated with high and low BMI were perceived as less healthy than other faces.

When both mouth curvature and facial BMI score (squared) were included in the same model, only the curvilinear relationship between facial BMI score and perceived health remained significant (b=-.35, t=-4.02, p<.001) whilst the predictive value of mouth curvature fell to a trend (b=-.15, t=-1.71, p=.090). Downward mouth curvature showed a trend to relate to higher facial BMI score (r(118)=.17, p=.072).

Actual BMI was also found to predict perceived health through linear and squared terms, although the relationship was mediated by face BMI scores .

Study 2: 2D images

Composite Images

Images displaying the average facial characteristics of the 10 faces rated highest and lowest in perceived health can be viewed in Figure 3.

Figure 3: Composite images illustrating the difference in facial characteristics of those rated low (left image, mean rating 3.5) and high (mean rating 5.7) in perceived health.

Skin Colour

Perceived health correlated with skin yellowness (b*: r(81)=.33, p=.003) but not skin redness (a*: r(81) =.11, p=.344) or lightness (L*: r(81) =-.19, p=.096). The correlation between perceived health and skin yellowness persisted after controlling for skin lightness (r=.28, df=78, p=.013).

Face Shape

Perceived health correlated with facial BMI score (r(79)=-.25, p=.023). As expected from the analysis of 3D face images, perceived health showed a stronger correlation with the square of

the facial BMI score (r(79)=-.35, p=.002). Facial BMI scores were correlated with actual BMI (r(79)=.55, p<.001) but did not mediate a relationship between actual BMI and perceived health . Perceived health correlated with upward mouth curvature (r(81)=.51, p<.001) but not eyelid openness (r(81)=.01, p=.953).

Entering skin yellowness, mouth curvature and facial adiposity into multiple regression with perceived health as the dependent variable revealed independent contributions to perceived health from skin yellowness (b=.22, t=2.11, p=.038), and from upward mouth curvature (b=.39, t=3.36, p=.001); (R2 =.28, overall model: F(3,75)=9.71, p<.001). This regression analysis failed to reveal an independent contribution of facial adiposity (b=-.06, t=-.46, p=.648) possibly because in this sample facial adiposity correlated with downward mouth curvature (r(79) =.53, p<.001).

Discussion

The composite 2D images (Figure 3) demonstrate multiple facial features associated with judgements of health. These include differences in skin colour, mouth curvature and shape related to weight. Analysis of objective image measurements confirmed that each of these cues was associated with impression of health. Mouth curvature was found to correlate with apparent health in both 2D and 3D images of faces; those with more downward turned mouths were rated as looking less healthy. We suggest that this relationship is driven by apparent sadness [51]. In line with this suggestion, the less healthy composite appears glum relative to the healthy composite. The extent to which one's eyes are open has previously been found to provide a cue to fatigue [51] but here we found no relation of eye openness to health judgements in either 2D or 3D facial images. The composite images do not appear to differ in fatigue.

A holistic measure of facial shape associated with adiposity was found to have a negative quadratic relationship to health judgements for both 2D and 3D face sets. That is, faces were judged to be less healthy as adiposity scores increased, but those with average adiposity scores were judged most healthy. For the 2D image set, skin yellowness but no other dimensions of skin colour (luminance and redness) was found to be positively associated with health judgements. Skin yellowness and face shape made independent contributions to judgements.

The quadratic relation between perceived health and measured facial adiposity in 2D and 3D found here was noted by Coetzee et al (2009) [22] when facial adiposity was estimated as perceived weight. The curvilinear relationship may be due to two aspects of health understood by evaluators. One component of understanding may reflect awareness that body weight increasing above average (or the medically recommended BMI of 25) is associated with negative health consequences including risk of cardiovascular disease and diabetes. A second component may reflect awareness that severe chronic and acute illnesses are associated with weight loss. Indeed, unintended weight loss can be a symptom of a variety of diseases including type 1 diabetes, cancer, and bacterial, viral or parasitic infection [62]. Health attributions triggered by low weight may reflect assumptions about a person's past or present condition. By contrast, attributions made to high weight could reflect the likelihood of current and future health problems (i.e. high weight may predispose cancer and heart disease in later life).

Facial BMI scores were associated with perceived health in both 3D and 2D face image sets with the relationship being stronger in the 3D face set. In 3D images, the dominance of facial adiposity in influencing judgements of health could be because additional shape information allowed more accurate judgements (the correlation between shape score and BMI was largest in 3D) or because the moving 3D images and lack of colour fostered an expectancy effect whereby raters assumed that they should use shape cues to inform health judgements.

Mouth curvature was found to be a more powerful predictor compared to facial BMI score in health judgements of 2D faces. Faces with more downturned mouths, which presumably looked sadder, were rated as less healthy. For the 3D faces, mouth curvature was again correlated with apparent health, yet for this set of faces it did not improve the model relative to facial BMI scores alone. Mouth curvature may have been a more salient cue in front-facing 2D images relative to rotating 3D faces. Alternatively, mouth curvature may vary across samples and across time for the same individual, hence the importance of mouth curvature for health judgements could be capricious and reflect sampling [63].

For the 2D face set, facial BMI scores correlated with downturned mouth curvature while for the 3D face set the same relationship showed a trend ($p=.070$). The association between mouth curvature and facial BMI scores we detected could arise because heavy jowls alter the real or apparent mouth shape. Alternatively, the link may arise because people with a heavier weight feel less comfortable in front of the camera. Since facial BMI scores showed a

curvilinear relationship to perceived health whilst mouth curvature showed a linear relationship, mouth curvature may have proportionally more importance for the faces of low weight individuals. By analogy, Fisher and colleagues (2014) showed that skin colour was less important for health judgements for high weight faces [23]. Hence while BMI and mouth curvature are somewhat related in the face sets we studied, they are logically separable and should be investigated for independent contributions in future studies.

Finally, in 2D images, colour was found to be a significant predictor of health judgements, independent of facial adiposity and mouth curvature. Although the effect of colour was weaker than that of mouth curvature, faces with more yellow colouration were judged as looking healthier. This is consistent with prior work demonstrating a reliable preference for yellowness in faces [38,45,47,61] but additionally illustrates that yellowness acts independently of adiposity and mouth curvature as a cue to health in unaltered facial images.

Our findings in 2D faces highlight the influence of colour and mouth curvatures (which is likely to be perceived as subtle expression) in judgements of health in a sample of young Caucasian adults. Our samples are relatively leaner and presumably healthier than the population at large. A minority of our sample would be considered overweight, with reference to their BMI and WHO guidelines (19% of our 3D, and 16% of our 2D samples) whilst the majority (64.6%) of the Scottish population from which they were drawn are reported to be overweight. In a more representative population sample, face shape (indices of weight and expression) and skin colour cues may have a different relative importance. A further limitation of the current work is that our 2D sample of images is limited to female faces. There were no sex-specific effects of the relationship between mouth curvature and facial adiposity to perceived health in the 3D sample; we therefore have no reason to believe that there should be in 2D faces either. We also do not anticipate any sex-specific effects of the relationship between colour and perceived health because the preference for carotenoid colouration of skin is not specific to the sex of the face or the rater [45]. There are no obvious theoretical grounds to suggest that the relative importance of colour and shape cues would differ by sex of the face or rater; nevertheless it is a question that could be addressed by future research.

Whilst it will be important to extrapolate these findings by testing the relative contribution of cues in older, heavier or more varied populations, the current findings highlight two important implications for the study of perceptual judgements. The first is that cues may be

utilised in a different manner depending upon the mode of stimulus presentation (e.g., 2D versus 3D), and this exposes the question of how cue utilisation differs in judgements from static images compared to video and in real life with natural facial movements. The second implication is that, even in standardised images with apparently neutral expressions, there will be subtle variation in apparent expression. Here we have demonstrated that not only is this subtle variation measureable but it is also influencing social judgements. Given that variation in apparent affect is prevalent in collections of neutrally posed faces [52], it is advisable that expression-related features are measured and considered in perceptual studies.

Finally, a wider implication exists for anyone who uses a 2D image to represent themselves in any context whereby social assessments and interactions may follow. Health judgements have been shown to influence judgements of both attractiveness and leadership ability [3,4]; so if you have a picture on an online dating site or a professional network profile you may wish to update it in light of our findings. An image with a healthy skin tone and a positive expression could improve your chances of love and success.

Additional Information

Acknowledgments

We thank Dengke Xiao for assistance with facial feature measurements and PCA analysis of face shape, and Anne Perrett for proof reading.

Ethics

All data collection was approved by UTREC and the School of Psychology and Neuroscience Ethics Committee, University of St Andrews. All participants provided informed written consent for their images and data to be used.

Data Accessibility

Data sets supporting this article can be accessed http://hdl.handle.net/10023/7924.
Software employed to measure and manipulate facial images is freely accessible online:
PsychoMorph (http://users.aber.ac.uk/bpt/jpsychomorph/)
MorphAnalyser (http://cherry.dcs.aber.ac.uk:8080/wiki/MorphAnalyser)

Authors' Contributions

Conception and design: All authors. Image and data collection: IH and ST. Statistical analysis: DP. Drafting the article: AH. Revising critically for important intellectual content: AH, IH and DP. Final approval of the version to be published: All authors.

Competing Interests

We have no competing interests.

Funding

AH is supported by a BBSRC studentship. DP was supported by a British Academy Wolfson Research Professorship.

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Appendix 2: Fruit and Vegetable Questionnaire

Think about your eating habits over the past week. How often did you eat each of the following foods? Remember breakfast, lunch, dinner, snacks and eating out. Check one box for each group.

- Fruit juice, like orange, apple, grape, fresh, frozen or canned (not sodas or other drinks

None	Once	2-3 times	4-6 times	1 per day	2 per day	3 per day	4 per day	5 per day	> 5 per day

- Any fruit, fresh or canned (not counting juice)

None	Once	2-3 times	4-6 times	1 per day	2 per day	3 per day	4 per day	5 per day	> 5 per day

- Vegetable juice, like tomato or carrot

None	Once	2-3 times	4-6 times	1 per day	2 per day	3 per day	4 per day	5 per day	> 5 per day

- Green salad

None	Once	2-3 times	4-6 times	1 per day	2 per day	3 per day	4 per day	5 per day	> 5 per day

- Potatoes, any kind, including baked, mashed or French fried

None	Once	2-3 times	4-6 times	1 per day	2 per day	3 per day	4 per day	5 per day	> 5 per day

- Vegetable soup or stew with vegetables

None	Once	2-3 times	4-6 times	1 per day	2 per day	3 per day	4 per day	5 per day	> 5 per day

- Any other vegetables, including string beans, peas, corn broccoli or any other kind

None	Once	2-3 times	4-6 times	1 per day	2 per day	3 per day	4 per day	5 per day	> 5 per day

Appendix 3: Godin Exercise Questionnaire

During a typical 7-day period, how many times on average do you do the following kinds of exercise for more than 15 minutes during your free time?

a. Strenuous Exercise (heart beats rapidly) _____ times

(e.g., running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling)

b. Moderate Exercise (not exhausting) _____ times

(e.g., fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing)

c. Mild Exercise (minimal effort) _____ times

(e.g., yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow-mobiling, easy walking)

Appendix 4: Stress Subscale

For the following series of questions, please read each statement and select the column which indicates how much the statement applied to you over the past week. There are no right or wrong answers. Do not spend too much time on any statement:

	Did not apply to me at all	Applied to me to some degree or some of the time	Applied to me to a considerable degree or a good part of the time	Applied to me very much or most of the time
I found it hard to wind down				
I tended to over-react to situations				
I felt that I was using a lot of nervous energy				
I found myself getting agitated				
I found in difficult to relax				
I was intolerant of anything that kept me from getting on with what I was doing				
I felt that I was rather touchy				

Appendix 5: Partial correlations from Chapter 3 controlling gender

Table 10: Partial correlations between predictor variables in cross sectional model controlling for gender (Study 1 Chapter 3).

	b*	L*	FV/day	Stress	Exercise	Alcohol
b*						
L*	-.436**					
FV/day	.347*	-.057				
Stress	-.254*	-.025	.116			
Exercise	.169	.048	.301*	.056		
Alcohol	-.141	-.095	-.215	-.023	.035	
% fat	-.118	-.064	-.055	-.069	-.181	.096

** $p<.001$; * $p<.05$

Table 11: Partial correlations between predictor variables in change model controlling for gender (Study 2 Chapter 3)

	b*	L*	FV/day	Stress	Exercise	Alcohol
b*						
L*	-.163					
FV/day	.193	-.110				
Stress	-.310*	-.045	-.065			
Exercise	-.065	.248	-.014	.177		
Alcohol	.181	.262	-.066	.020	-.061	
% fat	.010	-.036	.103	.182	-.161	.069

** $p<.001$; * $p<.05$

Appendix 6: International Physical Activity Questionnaire (IPAQ)

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the vigorous activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, aerobics, or fast bicycling?

_____ days per week

☐ No vigorous physical activities → Skip to question 3

2. How much time did you usually spend doing vigorous physical activities on one of those days?

_____ hours per day

_____ minutes per day

☐ Don't know/Not sure

Think about all the moderate activities that you did in the last 7 days. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.
_____ days per week

☐ No moderate physical activities → Skip to question 5

4. How much time did you usually spend doing moderate physical activities on one of those days?

_____ hours per day

_____ minutes per day

Don't know/Not sure

Think about the time you spent walking in the last 7 days. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the last 7 days, on how many days did you walk for at least 10 minutes at a time?

_____ days per week

☐ No walking → Skip to question 7

6. How much time did you usually spend walking on one of those days?

_____ hours per day

_____ minutes per day

Don't know/Not sure

The last question is about the time you spent sitting on weekdays during the last 7 days. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the last 7 days, how much time did you spend sitting on a week day?

_____ hours per day

_____ minutes per day

Don't know/Not sure

Appendix 7: Abbreviated Symptoms of Illness

For each of the symptoms listed below, participants first reported the frequency (Response options: 0 days, 1-3 days, 4-7 days, 8-14 days, 15-49 days, 50-60 days), and then if experienced, also the impact of symptoms on their daily activities (response options: did not interfere, slightly interfered, considerably interfered, severely interfered) with regard to the prior 2 months.

Symptoms

1. Sore throat
2. Coughing
3. Feeling exhausted or fatigued
4. Lightheaded faint or dizzy
5. Muscle aches or pain (not due to strenuous exercise)
6. Sinus problems
7. Nasal problems (runny nose, congestion)
8. Headaches
9. Fever
10. Changes in appetite
11. Swollen glands in neck

Appendix 8: Ethical Approval Letters

University of St Andrews

University Teaching and Research Ethics Committee
Sub-committee

3 December 2014

Ethics Reference No: *Please quote this ref on all correspondence*	PS11263
Project Title:	What Britain Eats
Researchers' Names:	Audrey Henderson and Dr Ross Whitehead
Supervisor:	Professor David Perrett

Thank you for submitting your application which was considered at the Psychology & Neuroscience School Ethics Committee meeting on the 18th November 2014. The following documents were reviewed:

1. Ethical Application Form 24/11/2014
2. Advertisement 24/11/2014
3. Participant Information Sheet 24/11/2014
4. Consent Form 24/11/2014
5. Debriefing Form 24/11/2014
6. Questionnaires 24/11/2014
7. Data Management Plan 24/11/2014

The University Teaching and Research Ethics Committee (UTREC) approves this study from an ethical point of view. Please note that where approval is given by a School Ethics Committee that committee is part of UTREC and is delegated to act for UTREC.

Approval is given for three years. Projects, which have not commenced within two years of original approval, must be re-submitted to your School Ethics Committee.

You must inform your School Ethics Committee when the research has been completed. If you are unable to complete your research within the 3 three year validation period, you will be required to write to your School Ethics Committee and to UTREC (where approval was given by UTREC) to request an extension or you will need to re-apply.

Any serious adverse events or significant change which occurs in connection with this study and/or which may alter its ethical consideration, must be reported immediately to the School Ethics Committee, and an Ethical Amendment Form submitted where appropriate.

Approval is given on the understanding that the 'Guidelines for Ethical Research Practice' https://www.st-andrews.ac.uk/utrec/guidelines/ are adhered to.

Yours sincerely

Convenor of the School Ethics Committee

Ccs Prof D. Perrett (Supervisor)
School Ethics Committee

School of Psychology & Neuroscience, St Mary's Quad, South Street, St Andrews, Fife KY16 9JP
Email: psyethics@st-andrews.ac.uk Tel: 01334 462071

University of St Andrews

University Teaching and Research Ethics Committee
Sub-committee

16 April 2015

Ethics Reference No: *Please quote this ref on all correspondence*	PS11441
Project Title:	Health and Health Judgements
Researchers' Names:	Audrey Henderson and Dr Ross Whitehead
Supervisors:	Professor David Perrett

Thank you for submitting your application which was considered at the Psychology & Neuroscience School Ethics Committee meeting on the 24th March 2015. The following documents were reviewed:

1.	Ethical Application Form	03/04/2015
2.	Participant Information Sheet	03/04/2015
3.	Consent Form	03/04/2015
4.	Debriefing Form	03/04/2015
5.	Questionnaire	03/04/2015
6.	Data Management Plan	03/04/2015

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Approval is given on the understanding that the 'Guidelines for Ethical Research Practice' https://www.st-andrews.ac.uk/utrec/guidelines/ are adhered to.

Yours sincerely

Convenor of the School Ethics Committee

Cc: Prof D. Perrett (Supervisor)
School Ethics Committee

School of Psychology & Neuroscience, St Mary's Quad, South Street, St Andrews, Fife KY16 9JP
Email: psyethics@st-andrews.ac.uk Tel: 01334 462071

University of St Andrews

University Teaching and Research Ethics Committee Sub-committee

1 November 2013

Ethics Reference No: *Please quote this ref on all correspondence*	PS10514
Project Title:	Individual Differences in Skin Colour Preferences
Researchers' Names:	Audrey Henderson, Lindsay Mackay and April Vellacott
Supervisor:	Professor David Perrett

Thank you for submitting your application which was considered at the Psychology & Neuroscience School Ethics Committee meeting on the 23rd October 2013. The following documents were reviewed:

1.	Ethical Application Form	31/10/2013
2.	Participant Information Sheet	31/10/2013
3.	Consent Form	31/10/2013
4.	Debriefing Form	31/10/2013
5.	Questionnaire	31/10/2013
6.	Data Management Plan	31/10/2013

The University Teaching and Research Ethics Committee (UTREC) approves this study from an ethical point of view. Please note that where approval is given by a School Ethics Committee that committee is part of UTREC and is delegated to act for UTREC.

Approval is given for three years. Projects, which have not commenced within two years of original approval, must be re-submitted to your School Ethics Committee.

You must inform your School Ethics Committee when the research has been completed. If you are unable to complete your research within the 3 three year validation period, you will be required to write to your School Ethics Committee and to UTREC (where approval was given by UTREC) to request an extension or you will need to re-apply.

Any serious adverse events or significant change which occurs in connection with this study and/or which may alter its ethical consideration, must be reported immediately to the School Ethics Committee, and an Ethical Amendment Form submitted where appropriate.

Approval is given on the understanding that the 'Guidelines for Ethical Research Practice' https://www.st-andrews.ac.uk/utrec/guidelines/ are adhered to.

Yours sincerely

Convenor of the School Ethics Committee

Ccs Prof D. Perrett (Supervisor)
School Ethics Committee

School of Psychology & Neuroscience, St Mary's Quad, South Street, St Andrews, Fife KY16 9JP
Email: psyethics@st-andrews.ac.uk Tel: 01334 462071

University of St Andrews

University Teaching and Research Ethics Committee
Sub-committee

17 September 2014

Ethics Reference No: *Please quote this ref on all correspondence*	PS11147
Project Title:	Exercise and Appearance
Researcher's Name:	Audrey Henderson
Supervisor:	Prof David Perrett

Thank you for submitting your application, which was considered at the Psychology & Neuroscience School Ethics Committee meeting on the 27th August 2014. The following documents were reviewed:

1.	Ethical Application Form	05/09/14
2.	Participant Information Sheets	05/09/14
3.	Participant Consent Form	05/09/14
4.	Participant Debriefing Form	05/09/14
5.	Data Management Plan	05/09/14
6.	Questionnaire	05/09/14
7.	Advertisement	05/09/14

The University Teaching and Research Ethics Committee (UTREC) approves this study from an ethical point of view. Please note that where approval is given by a School Ethics Committee that committee is part of UTREC and is delegated to act for UTREC.

Approval is given for three years. Projects, which have not commenced within two years of original approval, must be re-submitted to your School Ethics Committee.

You must inform your School Ethics Committee when the research has been completed. If you are unable to complete your research within the 3 three year validation period, you will be required to write to your School Ethics Committee and to UTREC (where approval was given by UTREC) to request an extension or you will need to re-apply.

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Approval is given on the understanding that the 'Guidelines for Ethical Research Practice' https://www.st-andrews.ac.uk/utrec/guidelines/ are adhered to.

Yours sincerely

Convenor of the School Ethics Committee

Cc: Prof David Perrett (Supervisor)
School Ethics Committee

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Email: psyethics@st-andrews.ac.uk Tel: 01334 462071

University of St Andrews

University Teaching and Research Ethics Committee
Sub-committee

17 September 2014

Ethics Reference No: *Please quote this ref on all correspondence*	PS11146
Project Title:	Team Sports: Fitness and Skin Colour
Researchers' Names:	Audrey Henderson and Rebecca Ijzendahl
Supervisor:	Prof David Perrett

Thank you for submitting your application, which was considered at the Psychology & Neuroscience School Ethics Committee meeting on the 27th August 2014. The following documents were reviewed:

1. Ethical Application Form 05/09/14
2. Participant Information Sheets 05/09/14
3. Participant Consent Form 05/09/14
4. Participant Debriefing Form 05/09/14
5. Data Management Plan 05/09/14
6. Questionnaire 05/09/14

The University Teaching and Research Ethics Committee (UTREC) approves this study from an ethical point of view. Please note that where approval is given by a School Ethics Committee that committee is part of UTREC and is delegated to act for UTREC.

Approval is given for three years. Projects, which have not commenced within two years of original approval, must be re-submitted to your School Ethics Committee.

You must inform your School Ethics Committee when the research has been completed. If you are unable to complete your research within the 3 three year validation period, you will be required to write to your School Ethics Committee and to UTREC (where approval was given by UTREC) to request an extension or you will need to re-apply.

Any serious adverse events or significant change which occurs in connection with this study and/or which may alter its ethical consideration must be reported immediately to the School Ethics Committee, and an Ethical Amendment Form submitted where appropriate.

Approval is given on the understanding that the 'Guidelines for Ethical Research Practice' https://www.st-andrews.ac.uk/utrec/guidelines/ are adhered to.

Yours sincerely

pp

Convenor of the School Ethics Committee

Cc Prof David Perrett (Supervisor)
School Ethics Committee

School of Psychology & Neuroscience, St Mary's Quad, South Street, St Andrews, Fife KY16 9JP
Email: psyethics@st-andrews.ac.uk Tel: 01334 462071

University of St Andrews

University Teaching and Research Ethics Committee
Sub-committee

17 September 2014

Ethics Reference No: *Please quote this ref on all correspondence*	PS11144
Project Title:	Prospective Illness Study
Researcher's Name:	Audrey Henderson
Supervisor:	Prof David Perrett

Thank you for submitting your application, which was considered at the Psychology & Neuroscience School Ethics Committee meeting on the 27th August 2014. The following documents were reviewed:

1.	Ethical Application Form	02/09/14
2.	Participant Information Sheet	02/09/14
3.	Participant Consent Form	02/09/14
4.	Participant Debriefing Form	02/09/14
5.	Data Management Plan	02/09/14
6.	Questionnaire	02/09/14

The University Teaching and Research Ethics Committee (UTREC) approves this study from an ethical point of view. Please note that where approval is given by a School Ethics Committee that committee is part of UTREC and is delegated to act for UTREC.

Approval is given for three years. Projects, which have not commenced within two years of original approval, must be re-submitted to your School Ethics Committee.

You must inform your School Ethics Committee when the research has been completed. If you are unable to complete your research within the 3 three year validation period, you will be required to write to your School Ethics Committee and to UTREC (where approval was given by UTREC) to request an extension or you will need to re-apply.

Any serious adverse events or significant change which occurs in connection with this study and/or which may alter its ethical consideration must be reported immediately to the School Ethics Committee, and an Ethical Amendment Form submitted where appropriate.

Approval is given on the understanding that the 'Guidelines for Ethical Research Practice' https://www.st-andrews.ac.uk/utrec/guidelines/ are adhered to.

Yours sincerely

pp

Convenor of the School Ethics Committee

Cos Prof David Perrett (Supervisor)
School Ethics Committee

School of Psychology & Neuroscience, St Mary's House, South Street, St Andrews, Fife KY16 9JP

University of St Andrews
from first to foremost

600 YEARS
1413 – 2013

Project Title	Prospective Illness Study
Researcher's Name	Audrey Henderson
Supervisor	Professor David Perrett
Department/Unit	School of Psychology & Neuroscience
Ethical Approval Code (Approval allocated to Original Application)	PS11144
Original Application Approval Date	02 September 2014
Amendment Application Approval	30 September 2014

Ethical Amendment Approval

Thank you for submitting your amendment application which was considered by the Psychology & Neuroscience School Ethics Committee on the 30th September 2014. The following documents were reviewed:

1. Ethical Amendment Application Form 30/09/2014
2. Contact Email 30/09/2014
3. Participant Information Sheet 30/09/2014
4. Debriefing Form 30/09/2014

The University Teaching and Research Ethics Committee (UTREC) approves this study from an ethical point of view. Please note that where approval is given by a School Ethics Committee that committee is part of UTREC and is delegated to act for UTREC.

Approval is given for three years from the original application only. Ethical Amendments do not extend this period but give permission to an amendment to the original approval research proposal only. If you are unable to complete your research within the original 3 three year validation period, you will be required to write to your School Ethics Committee and to UTREC (where approval was given by UTREC) to request an extension or you will need to re-apply. You must inform your School Ethics Committee when the research has been completed.

Any serious adverse events or significant change which occurs in connection with this study and/or which may alter its ethical consideration, must be reported immediately to the School Ethics Committee, and an Ethical Amendment Form submitted where appropriate.

Approval is given on the understanding that the 'Guidelines for Ethical Research Practice' (http://www.st-andrews.ac.uk/media/UTRECguidelines%20Feb%2008.pdf) are adhered to.

Yours sincerely

Convenor of the School Ethics Committee

Cc: School Ethics Committee
Professor D Perrett (Supervisor)

School of Psychology & Neuroscience, St Mary's Quad, South Street, St Andrews, Fife KY16 9JP
Email: psyethics@st-andrews.ac.uk Tel: 01334 462071

BESLUT

Dnr: 2014/1946-31/2
Sökande: Karolinska Institutet
Behörig företrädare: Bo Melin
Projekt: Endotoxininducerad inflammation, beteendereaktioner och prediktorer för individuella skillnader
Forskare som genomför projektet: Mats Lekander

Nämnden har vid sammanträdet den 21/1 2015 lämnat över till den vetenskaplige sekreteraren att avgöra ärendet sedan kompletteringar gjorts.

Sedan sökanden kommit in med begärda kompletteringar fattar den vetenskaplige sekreteraren följande

BESLUT
Ansökan godkännes

2015 -02- 06

På nämndens vägnar

Pierre Lafolie
Vetenskaplig sekreterare

Beslut expedierat till behörig företrädare
Kopia för kännedom till ansvarig forskare

Postadress	Besöksadress	Telefon	Fax	E-post	Hemsida
PE 280 171 77 STOCKHOLM	Nobels väg 9 Solna	08 524 870 00 (vx)	08 524 856 90	kansli@regionalt.etikprovning.se	www.epn.se

University of St Andrews | FOUNDED 1413

University Teaching and Research Ethics Committee

01 August 2016

Dear Audrey

Thank you for submitting your ethical application which was considered by the School of Psychology & Neuroscience Ethics Committee on 1st August 2016; the following documents have been reviewed:

1. Front Page of UTREC Ethical Application Form
2. Cover Letter to School of Psychology & Neuroscience SEC
3. Copy of Ethical Approval granted by Karolinska Institutet
4. Copy of Ethical Application approved by Karolinska Institutet

The School of Psychology & Neuroscience Ethics Committee has been delegated to act on behalf of the University Teaching and Research Ethics Committee (UTREC) and has granted this application ethical approval. The particulars relating to the approved project are as follows -

Approval Code:	PS12322	**Approved on:**	01/08/2016	**Approval Expiry:**	01/08/2021
Project Title:	Skin colour changes with experimentally induced sickness				
Researchers:	Audrey Henderson				
Supervisor:	Professor David Perrett				

Approval is awarded for five years. Projects which have not commenced within two years of approval must be re-submitted for review by your School Ethics Committee. If you are unable to complete your research within the five year approval period, you are required to write to your School Ethics Committee Convener to request a discretionary extension of no greater than 6 months or to re-apply if directed to do so, and you should inform your School Ethics Committee when your project reaches completion.

If you make any changes to the project outlined in your approved ethical application form, you should inform your supervisor and seek advice on the ethical implications of those changes from the School Ethics Convener who may advise you to complete and submit an ethical amendment form for review.

Any adverse incident which occurs during the course of conducting your research must be reported immediately to the School Ethics Committee who will advise you on the appropriate action to be taken.

Approval is given on the understanding that you conduct your research as outlined in your application and in compliance with UTREC Guidelines and Policies (http://www.st-andrews.ac.uk/utrec/guidelinespolicies/). You are also advised to ensure that you procure and handle your research data within the provisions of the Data Provision Act 1998 and in accordance with any conditions of funding incumbent upon you.

Yours sincerely

Convener of the School Ethics Committee

cc Professor David Perrett (Supervisor)

School of Psychology & Neuroscience, St Mary's Quad, South Street, St Andrews, Fife KY16 9JP
Email: psyethics@st-andrews.ac.uk Tel: 01334 462071

University of St Andrews

University Teaching and Research Ethics Committee Sub-committee

17 September 2014

Ethics Reference No: *Please quote this ref on all correspondence*	PS11145
Project Title:	Perceived Health
Researchers' Names:	Audrey Henderson and Xiaobo Zhang
Supervisor:	Prof David Perrett

Thank you for submitting your application, which was considered at the Psychology & Neuroscience School Ethics Committee meeting on the 27th August 2014. The following documents were reviewed:

1. Ethical Application Form 02/09/14
2. Participant Information (Online) 02/09/14
3. Participant Consent (Online) 02/09/14
4. Participant Debriefing(Online) 02/09/14
5. Data Management Plan 02/09/14
6. Questionnaire 02/09/14

The University Teaching and Research Ethics Committee (UTREC) approves this study from an ethical point of view. Please note that where approval is given by a School Ethics Committee that committee is part of UTREC and is delegated to act for UTREC.

Approval is given for three years. Projects, which have not commenced within two years of original approval, must be re-submitted to your School Ethics Committee.

You must inform your School Ethics Committee when the research has been completed. If you are unable to complete your research within the 3 three year validation period, you will be required to write to your School Ethics Committee and to UTREC (where approval was given by UTREC) to request an extension or you will need to re-apply.

Any serious adverse events or significant change which occurs in connection with this study and/or which may alter its ethical consideration must be reported immediately to the School Ethics Committee, and an Ethical Amendment Form submitted where appropriate.

Approval is given on the understanding that the 'Guidelines for Ethical Research Practice' https://www.st-andrews.ac.uk/utrec/guidelines/ are adhered to.

Yours sincerely

Convenor of the School Ethics Committee

Cc: Prof David Perrett (Supervisor)
School Ethics Committee

School of Psychology & Neuroscience, St Mary's Quad, South Street, St Andrews, Fife KY16 9JP
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